Symposia Biologica Hungarica 23

FISH, PATHOGENS AND ENVIRONMENT IN EUROPEAN POLYCULTURE

Akadémiai Kiadó, Budapest

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23

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Proceedings
of an International Seminar
June 23–27, 1981
Szarvas, Hungary

Edited by
J. OLÁH

(Symposia Biologica Hungarica 23)

The volume presents the Proceedings of an International Seminar devoted to the combined problems of fish health and adverse environmental effects. These problems have come into the limelight along with the rapid development of the European warm water fish culture.

The complexity of fish, pathogens and environment necessitated the organization of interdisciplinary research teams. The results were presented at the international forum of the Seminar. Special attention was paid to "gill-necrosis" due to its importance in the carp culture practice of Central and Eastern Europe.

Separate chapters of the volume deal with the viral, bacterial and parasitic diseases of fish, as well as with the environmental effects and with the results of immunology.

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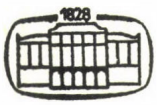

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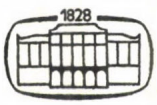

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CONTENTS

PARASITES OF FISHES

ENVIRONMENTAL EFFECTS

IMMUNOLOGY

LIST OF PARTICIPANTS

AHNE W.
Institut für Zoologie und Hydrobiologie der Universität, München, BRD

ANDERSON D.P.
National Fish Health Research Laboratory, Kearneysville, West Virginia, USA

ANTYCHOWICZ J.
Fish Health Research Department of Veterinary Institute, Pulawy, Poland

BAKONYI A.
Veterinary Station of Csongrád County, Szeged, Hungary

BÁNDI P.
Veterinary Station of Pest County, Gödöllő, Hungary

BÉKÉSI L.
Central Veterinary Institute, Budapest, Hungary

BODNÁR A.
Veterinary Station of Nyiregyháza, Hungary

BOROS G.
Department of Pathology, Veterinary University, Budapest, Hungary

BOROSS L.
József Attila University, Department of Biochemistry, Szeged, Hungary

BOYADZIEV N.M.
"Hristo Botev" Bl, Sofia, Bulgaria

CIRKOVIC M.
Veterinary Institute, Novi Sad, Yugoslavia

CSABA GY.
Central Veterinary Institute, Budapest, Hungary

CSÁVÁS I.
Fisheries Research Institute, Szarvas, Hungary

DYKOVÁ I.
Institute of Parasitology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

FARKAS J.
Fisheries Research Institute, Szarvas, Hungary

FERCEC N.
Fish Culture Institute, Nasicka Breznica, Yugoslavia

FIJAN N.
Department for Biology and Pathology of Fish and Bees, Veterinary Faculty, Zagreb, Yugoslavia

GYÖNGYÖSI ZS.
Fisheries Research Institute, Szarvas, Hungary

GYÖRE K.
Fisheries Research Institute, Szarvas, Hungary

HAVLIKOVA J.
Fisheries Research Institute, Vodnany, Czechoslovakia

JENEY G.
Fisheries Research Institute, Szarvas, Hungary

JENEY ZS.
Fisheries Research Institute, Szarvas, Hungary

JEREMIC S.
Veterinary Institute of Serbia, Novi Sad, Yugoslavia

JUHÁSZ-BUCSEK M.
Central Veterinary Institute, Budapest, Hungary

KISKÁROLY M.
Ivana markovica Irca, Sarajevo, Yugoslavia

KOVÁCS-GAYER É.
Central Veterinary Institute, Budapest, Hungary

KRASZNAI Z.
Fisheries Research Institute, Budapest, Hungary

KULOW H.
Institute für Binnenfischerei, Berlin-Friedrichshagen, GDR

LÁNG M.
Warmwater Fish Hatchery, Százhalombatta, Hungary

LOM J.
Institute of Parasitology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

LUCKÝ Z.
Veterinary College, Brno, Czechoslovakia

MALIK E.
Fisheries Research Institute, Szarvas, Hungary

MÁRIÁN T.
Fisheries Research Institute, Szarvas, Hungary

MATAŠIN Z.
Department for Biology and Pathology of Fish and Bees, Veterinary Faculty, Zagreb, Yugoslavia

MATSKÁSI I.
Research Institute for Animal Hygiene, Hungarian Academy of Sciences, Budapest, Hungary

MOHOS A.
Jászkarajenő, Hungary

MOLNÁR K.
Research Institute for Animal Hygiene, Hungarian Academy of Sciences, Budapest, Hungary

MUSSELIUS V.A.
All-Union Research Institute of Fisheries, Moscow Region, Rybnoe, USSR

MÜLLER F.
Fisheries Research Institute, Szarvas, Hungary

NAGY I.
Dömsöd, Hungary

NEMCSÓK J.
Department of Biochemistry, József Attila University, Szeged, Hungary

OCVIRK J.
Institute of Biotechnology for Veterinary Sciences, Edward Kardelj University, Ljubljana, Yugoslavia

OLÁH J.
Fisheries Research Institute, Szarvas, Hungary

O. TÓTH E.
Fisheries Research Institute, Szarvas, Hungary

OSADCHAYA E.F.
Fish Culture Research Institute of Ukraine, Kiev, USSR

PETRINEC Z.
Department for Biology and Pathology of Fish and Bees, Veterinary Faculty, Zagreb, Yugoslavia

PRIHODA J.
Zinila, Safárikova, Czechoslovakia

PROST M.
Akademicka 12, Lublin, Poland

PRIGLI M.
Veterinary Station of Csongrád County, Szeged, Hungary

PÓCSI L.
Agricultural University of Debrecen, Debrecen, Hungary

PUSZTAI M.
Central Veterinary Institute, Budapest, Hungary

REHULKA J.
Fish Research Institute, Ostrava, Czechoslovakia

ROJIK I.
Department of Comparative Zoology, József Attila University, Szeged, Hungary

SCHRECKENBACH K.
Institut für Binnenfischerei, Berlin-Friedrichshagen, GDR

SHCHELKUNOV I.S.
All-Union Research Institute of Fisheries, Moscow Region, Rybnoe, USSR

SHESTAKOVSKAYA E.V.
Azovian Fish Health Research Institute, Rostov-na-Donu, USSR

SIWICKI A.
Institute Inland Fishery, Zabieniec, Paseczno, Poland

SNOJ N.
Institute of Biotechnology for Veterinary Sciences, Edward Kardelj University, Ljubljana, Yugoslavia

SÖVÉNYI J.
Research Institute for Animal Hygiene, Hungarian Academy of Sciences, Budapest, Hungary

SPANGENBERG R.
Institut für Binnenfischerei, Berlin-Friedrichshagen, GDR

SPRINGER J.
Veterinary Station of Bács-Kiskun County, Kecskemét, Hungary

STUDNICKA-SIWICKA M.
Institute Inland Fishery, Zabieniec, Piaseczno, Poland

SVOBODOVA Z.
Fisheries Research Institute, Vodnany, Czechoslovakia

SZABÓ P.
Fisheries Research Institute, Szarvas, Hungary

SZAKOLCZAI J.
Central Veterinary Institute, Budapest, Hungary

SZITÓ A.
Fisheries Research Institute, Szarvas, Hungary

STRELKOV Yu. A.
National Institute for Research of Lakes and Rivers, Leningrad, USSR

TCHIKOVA V.D.
NPO "Veterinarno del", Sofia, Bulgaria

TURKOVICS O.
Association of Cooperative Fisheries, Budapest, Hungary

VERBICKAYA I.N.
Ministry for Fish Culture of USSR, Moscow, USSR

WEIROWSKI F.
Institut für Binnenfischerei, Berlin-Friedrichshagen, GDR

ZELAZNY J.
Fish Health Research Department of Veterinary Institute, Pulawy, Poland

ŽITŇAN R.
Helminthological Institute of the Slovak Academy of Sciences, Košice, Czechoslovakia

OPENING ADDRESS

The production of freshwater fish has been gaining an increasing importance in recent years. The one-time inexhaustible stock of fish has stagnated during the past decades, though demands have been growing steadily. According to an FAO survey, the protein-rich fish meat would be the most suitable way of satisfying the food requirement of millions of starving people.

More and more can be heard nowadays about the harmful effects and dramatic pollution affecting the seas. These factors are seriously threatening the life in the waters. Maritime countries have extended their territorial waters thereby ousting fishing fleets. The energy-crisis also had a very unfavourable impact on marine fishing. This situation directed the attention to the necessity of increasing the production of inland fisheries. During the past few years, the inland fisheries have been increasing their share in fish production.

The prospects for expanding fresh-water production are especially good in the developing countries, but rapid progress can be attained by introducing intensive methods in many European countries as well. So, the developed countries also strive to increase inland fish production, since the change in the way of life is accompanied by changes in the structure of meat consumption, and an increasing demand for fish meat.

Fish production of Hungary has been around 35 thousand tons in the past few years. This should not be undervalued, since our country does not deal with marine fishing. The growing fish production comes from natural waters, ponds and partly from the intensive fish farms. Fish farming in Hungary dates back almost to a hundred years.

The main trend of the past years was to intensify Hungarian fish production and the current practice of fish farming is to stock two- or three-fold of the population in polycultural systems.

Hatching, and nursing fish fry have gradually gained place in our country. At the same time, the provision of standard stock material may be dangerous.

There are great possibilities in our country for utilization of geothermic waters and effluents of power stations. To produce more fish, we have to use up-to-date methods, - e.g. cage culture - and to exploit water reservoirs, strip-mine-lakes, etc.

Intensive fish-production should be accompanied by radical improvement of sanitary conditions. Unfortunately, this does not always follow the advance of technology and often results in losses instead of enhancement of production. Two cases can be mentioned in Hungary. At the time of quick raising of fish production of the 1950s, dropsy occurred and was followed by the ichthyophthiriases in the 1960s.

Diseases can spread partly because of the favourable changes of biotopes for pathogens. Other diseases are caused by introduced pathogens. Environmental stress factors /insufficient quality of water, low O_2 content, toxic materials, etc./ are also important.

Losses can be reduced by improving the hygienic conditions of the ponds, erforcing stricter animal health measures and advancing fish health research.

Methods of intensive breeding of salmonids have old traditions. Earlier, the gross mortality of these species was observed which could be eliminated mainly by improving environmental conditions and by maintaining sanitary terms which could be ensured with salmonids kept in concrete basins.

Fish farms producing carp in warm water have greater difficulty in maintaining hygienic conditions and breeding disease-resistant fish. The actual veterinary measures in these places should be confined to quick diagnosis, effective intervention and elimination of obligate pathogens.

In Hungary, research in fish health protection has relatively old traditions. As early as in 1905, Professor Rátz established a laboratory for fish pathology at the Veterinary College.

He described the most important diseases as well as the possibilities for prevention and therapy. Unfortunately, this early initiation was not followed by further steps.

After World War II, co-workers at the Fisheries Research Institute at Gödöllő tried to cope with the fish diseases appearing with the boom of fish production. In 1957, veterinary organizations took over the task. In the 1950s, courses were launched on fish-breeding, and fish-pathology at the Veterinary College of Budapest. Compulsory tutorials were introduced in 1958. At the same time, diagnostic work began at the Animal Health Institute, Budapest, and later at the regional animal health centres, as well. Research on fish parasites was initiated in the early 1960s. As a result of thorough research and diagnostic work, dropsy, ichthyophthiriases, dactylogyrosis of carp have been significantly repressed, and methods for controlling parasites introduced into the country with herbivorous fishes were also worked out. Procedures for prevention and therapy are included in the instructions used country-wide to complete the technology.

Beside general diagnostic work, pathological, virological, bacteriological and hydrochemical research is being done at the National Animal Health Institute. Successful parasitological research has long been conducted at the Veterinary Research Institute of the Hungarian Academy of Sciences. The Biological Department of the Fisheries Research Institute, Szarvas has been doing research in bacteriology, parasitology, hydrobiology, hydro-chemistry and environmental hygiene.

The diagnostic network - besides the work done at the National Animal Health Institute - is based on the work of specialists at the regional animal health services and fish farms.

The majority of diseases caused by parasites are known, prevention, however, is far from being solved. We have a lot to do, first of all, in the field of pathology, and therapy of sporozoan unicellulars.

Much less is known, however, about bacterial diseases. Erythrodermatitis of carp and Flexibacter infections in the hatcheries are the only diseases about which we have sufficient data. The fact, however, that acute dropsy, known as "spring viraemia" and attributed to viruses can be well-treated with

antibiotics means that the role of bacteria, as secondary infecting agents, cannot be neglected either, and should be dealt with. We have less success in the diagnosis of viral diseases. So far, only viruses causing spring viraemia and epithelioma myxomatosa could be isolated.

Unfortunately, we have scanty information on the etiology of the most important diseases as gill necrosis, swim bladder inflammation, which endanger the fish-stock in Hungary, and the neighbouring countries. An international co-operation would be desirable to solve these problems.

Diseases introduced into Hungary prove that fish pathogens can spread freely. Successful prevention and treatment can only be achieved by being up to date with advances in research. A good example of this is that our specialists could take precautions against Bothriocephalus introduced into Europe along with herbivorous fishes. Though the scientists of different countries were concerned with and working on similar problems, there was no possibility for quick information exchange.

These problems could not be solved with the fact that international organizations, like EIFAC or OIE included them into their projects as the most urgent questions. Information exchange and cooperation was also hindered, on one hand, by the fact that experts working in animal health organizations regarded fish health as a special field. On the other hand, experts working on questions of fish health - as parasitologists, epizoologists, hydrochemists, etc. - were not aware of each other's work either and published their results in a wide range of journals.

The identification and spread of viral diseases, as well as the appearance of diseases of unknown etiology emphasized the fact that there is no perspective in isolated research, that the specialists working in different fields should know each other's work. They realized the importance of bi- and multilateral co-operation and the necessity of a competent, international organization to mediate communication.

The first steps in this field were made in 1975, when the meeting of COPRAQ was organized in Zagreb, followed by the next successful ones held in Brest, 1977 and Munich, 1979.

At the COPRAQ meeting of 1979, an international organization assembling specialists of fish and shellfish diseases, the EAFP /European Association of Fish Pathologists/ was founded which has been functioning successfully.

These COPRAQ meetings, however, had two shortcomings: /1/ Due to financial difficulties, the socialist countries were represented only by Yugoslavian and Hungarian experts. /2/ According to the interest of the majority, most of the subjects discussed were about diseases of cold-water fishes. Such problems as gill necrosis and swim-bladder inflammation were not put on the agenda at all.

Realizing the importance of scientific collaboration, first of all in solving the problems of etiology of gill necrosis, the Fisheries Research Institute backed by the veterinary organizations and authorities, decided to organize an international seminar on the most important diseases of fresh-water fish. This seminar is devoted to providing an opportunity for scientists from socialist countries to report on their results and to establish new scientific contacts. The aim of the seminar, beside the scientific discussions, is to find out how the countries which have not actively been working in EAFP can join the scientific work of the organization at the same time remaining financially independent.

I wish every scientist to contribute to the development and progress of this branch of science, to clarify differences and collaborate in the prevention of fish diseases mutually beneficient to mankind.

Lajos Dénes

Deputy Minister for
Food and Agriculture

VIRAL DISEASES

Symposia Biologica Hungarica 23 (1984)

IMPORTANT VIRAL DISEASES IN EUROPEAN FISH CULTURES

W. AHNE

Institute for Zoology and Hydrobiology,
University of Munich, Munich, FRG

ABSTRACT

Mainly four viral diseases cause economical losses in European fish cultures

- Infectious pancreatic necrosis of trout /IPN/
- Pike fry rhabdovirus disease /PFR/
- Spring viraemia of carp /SVC/
- Viral haemorrhagic septicaemia of trout /VHS/.

Besides the listed diseases two diseases occurring in carp populations

- Epithelioma papillosum of carp
- Gill necrosis of carp

may have a suspected viral aetiology also.

The agents, the pathological signs of the fish as well as control and diagnosis of the diseases are described.

INTRODUCTION

Losses due to viral diseases of cultured fishes have increased during the last years. A major factor in the spread of causal viruses is the transfer of live carrier fish and perhaps contaminated eggs. Therefore, some fish health control regulations have been legislated by several European countries.

In European fish cultures four viral diseases are mainly responsible for economic losses, moreover two additional diseases, probably caused by viruses too, attack carp /Table 1/.

Table 1 Important viral diseases in European fish culture and diseases with suspected viral etiology

Disease	Virus	Virusgroup	Fish affected
Infectious pancreatic necrosis	IPN (at least 3 subtypes)	Birna 	Salmonids and a variety of non - salmonid species
Pike fry rhabdovirus	PFR	Rhabdo 	Pike (grass carp, tench)
Spring viremia of carp	SVCV		Carp and perhaps other cyprinids
Viral hemorrhagic septicemia	VHS (3 subtypes)		Salmonids (greyling, pike)
* Epithelioma papillosum of carp	?	Herpes ?	Carp and other cyprinids
* Gill necrosis of carp	?	Herpes ?	Carp

* suspected viral etiology

Infectious pancreatic necrosis /IPN/

The IPN is a geographically widespread viral disease of young fish of many salmonid species /brook trout, brown trout, rainbow trout, Atlantic salmon, chinook salmon, coho salmon and others/.

The typical victims of the acute infection are fry and fingerling. Fish more than 6 months' old become virus carriers. Besides salmonids the virus has been isolated from a variety of non-salmonid fish /Hill 1977, Ahne 1978a, 1980/.

Clinical signs are dark colouration, distended abdomen and spiral swimming. Internally, the pancreatic tissues show hemorrhages, pale liver and spleen and the digestive tract is usually free of food but a yellowish mucus is present in the stomach and gut. Histologically, destruction of exocrine pancreatic acinar tissue is evident.

Under hatchery conditions the mortality is usually high /50-80 %/. Survivors of an epizootic become carriers for their life time and the virus is shed by faeces. The agent is trans-

mitted by several vectors /carrier-fish, water, birds, equipment/. A vertical transmission with eggs from carrier brood stock has been observed also. Unfortunately, eggs contaminated with the virus cannot be adequately disinfected /Bullock et al.1976/. The causal agent is an unenveloped icosahedral virus containing double-stranded RNA. The virion is 55-75 nm in diameter and has 92 capsomeres. The IPN-virus could not be included in any of the present virus genera and a new group which is called Birna-virus is suggested for the IPN and IPN-like viruses /Dobos et al. 1979, Fig. 1/.

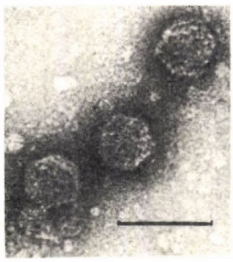


Figure 1 Electron photomicrograph of IPN virus negatively stained with PTA /calibration bar represents 100 nm/

All European isolates of IPN belong to the IPN-Sp /90 %/ and IPN-Ab /10 %/ subtypes /Jørgensen 1974/. The American reference strain VR-299 has not been isolated in Europe so far. As shown in Table 2 each of the subtypes cross-react in the neutralization test but differences in the degree of neutralization are significant.

Vaccines for control of the disease are not available at present. Eradication of infected fish, disinfection of the entire hatchery, the use of eggs from virus-free broodfish and the use of springwater are effective control measures.

IPN can be diagnosed either by isolation and identification of the virus or by immunofluorescence on cryostate thin sections. Seroepizootiologic studies can be done by serum neutralization tests. However, due to the fact that IPN-virus which has been passed sometimes in tissue cultures is neutralized by a factor /6S/ present in the serum of IPN-free trout /Dorson et al.1975/, the reference virus used in the test must be 6S-resistant.

Table 2 Cross-neutralization between the major IPN-subtypes /after Underwood et al. 1977/

Antiserum	IPN-subtypes IPN-Sp	IPN-Ab	IPN-VR 299
IPN-Sp	360 000×	3 500	5 400
IPN-Ab	2 600	1 200 000	3 300
IPN-VR 299	5 800	2 400	760 000

× (reciprocal of antiserum dilution giving 50% plaque count reduction)

This can be an IPN-virus which has been passed through the tissue culture only once or a virus that has been grown in tissue culture in the presence of normal trout serum /Hill personal communication/.

Diseases in cultured fish caused by rhabdoviruses

There are mainly three rhabdovirus diseases occurring in European fish cultures /Table 1/.

Generally speaking, the rhabdoviruses are bullet-shaped particles measuring 60-80 nm x 120-180 nm /Fig. 2/.

The virion consists of an outer surface with spikes /glycoprotein/, an envelope /lipid/ and a central core with single stranded RNA. SVCV and PFR share a polypeptide composition similar to rabies virus.

Rhabdoviruses are endotheliotropic agents and cause systemic infections in fishes which lead mainly to disturbances in the metabolism of salts and water.

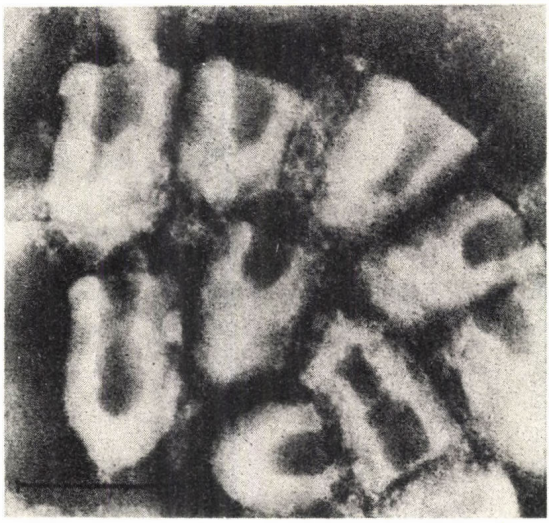


Figure 2 Electron photomicrograph of a rhabdovirus isolated from cyprinid fish. PTA negative staining. /Calibration bar represents 100 nm/

Pike fry rhabdovirus disease /PFRD/

PFRD is a contagious disease of juvenile Northern pike which has been observed in Holland only till now. In cultured pike fry the disease produces high mortality mainly in springtime. Affected pike show red swollen areas on both sides of the fish body and exophthalmus and hemorrhages on gills are present.

The infection can be transmitted via water or with contaminated eggs /Bootsma et al. 1975/. The agent /Pike fry rhabdovirus, PFR/ is serologically distinct from the other fish-rhabdovirus reference strains /Table 3/.

However, two rhabdoviruses isolated from grass-carp /Ctenopharyngodon idella/ and one isolate from tench /Tinca tinca/ show a high degree of relatedness with PFR by means of neutralization tests /Ahne, unpubl./. Fish besides pike may be carriers and reservoirs. Effective control measures are disinfection with jodophors, eradication of infected fish.

Spring viraemia of carp /SVC/

Spring viraemia of carp is an acute systemic viral infection of common carp /Cyprinus carpio/ in Europe. Prior to the

Table 3 Cross-neutralization between some fish rhabdoviruses /after Hill et al. 1975/

SVCV = Spring viraemia of carp virus; PFR = Pike fry rhabdovirus; IHNV = Virus of infectious haematopoietic necrosis; VHS = Virus of haemorrhagic septicaemia of trout

	Viruses			
Antiserum	SVCV	PFR	IHNV	VHS (F_1)
SVCV	6500 ×	20	20	< 10
PFR	33	200	27	< 10
IHNV	25	27	1700	< 10
VHS (F_1)	< 10	< 10	< 10	2300

×(reciprocal of antiserum dilution giving 50% plaque count reduction)

isolation of the viral agent /Spring viraemia of carp virus, SVCV; rhabdovirus carpio, RVC/ by Fijan et al. /1971/ the disease-complex was known as "infectious dropsy of carp".

SVCV has the characteristics of the rhabdoviruses and the agent is pathogen to carp of all ages and perhaps other Cyprinids. The disease becomes manifest when water temperatures rise in the springtime. Moribund fish show dark colouration, low respiration, exophthalmia, inflamed and oedematous vent, pale gills and haemorrhages in the skin. Internally, there are frequently haemorrhages in the viscera and air bladder, peritonitis with serous or haemorrhagic exudate is mostly present in acute cases.

The virus is shed by faeces and possible urine /Fig. 3/. Blood sucking parasites /Piscicola geometra and Argulus foliaceus/ are vectors for SVCV /Ahne 1979/. The virus is transmit-

ted by water route and replication of SVCV was demonstrated first in the gills /Ahne 1978b/.

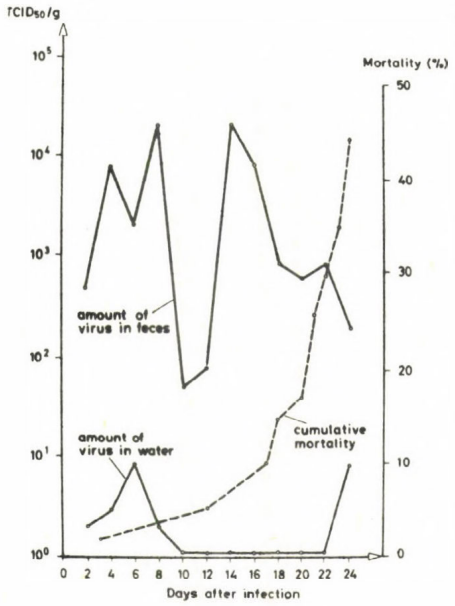


Figure 3 Shedding of SVCV by infected carp and cumulative mortality /13°C/

Infected carp kept at a water temperature above 15°C develop humoral antibodies and the fish show a long lasting immunity. In contrast, infected carp which were kept at water temperatures lower than 15°C exhibited about 90 % mortality in 30 days /Fig. 4/.

As shown in Fig. 5 carp which were immunized with SVCV showed a protective immunity to a reinfection with a virulent SVCV strain even at lower temperatures /10°C/.

From this point of view the immunoprophylaxis seems to be an effective approach for the control of SVC.

For diagnosis the agent can be detected either by isolation or by immunofluorescence of thin section of organs of infected fish. For epizootiologic surveys serum neutralization tests showed to be an effective method /Kölbl and Kainz 1977, Wizigmann et al. 1980a/.

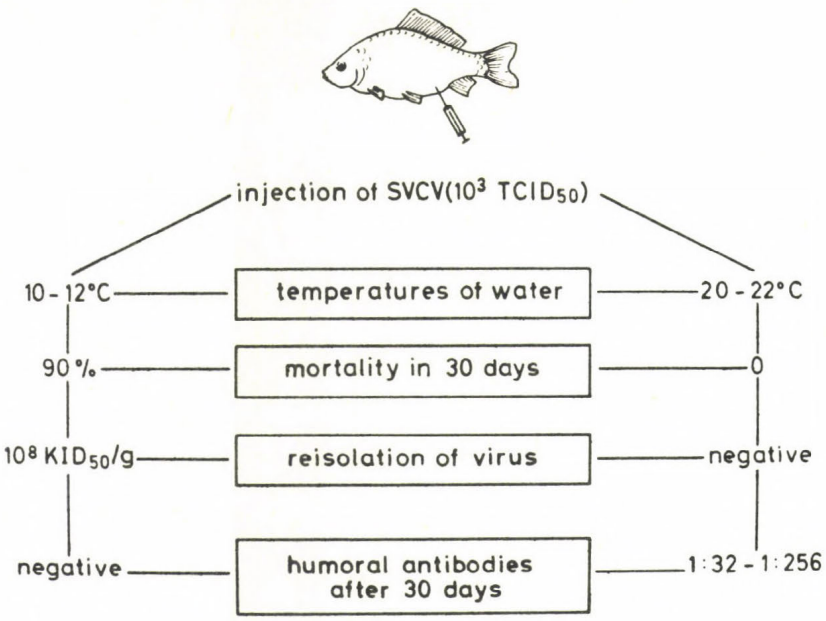
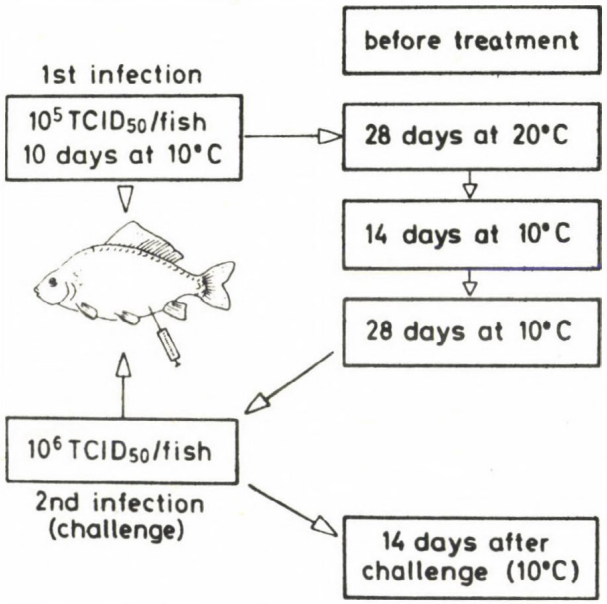


Figure 4 Influence of water temperatures on SVCV infection in carp

Viral haemorrhagic septicaemia /VHS/

The VHS is regarded as a highly contagious viral disease of trout of the disease does occur in pike and in greyling also /Wizigmann et al. 1980b, Meier and Jørgensen 1980/. Three subtypes of VHS have been described /Table 4/. Jørgensen /1974/ differentiated the VHS-subtypes VHS-1 /=Egtvedvirus/ and VHS-2. In France de Kinkelin and Le Berre /1977/ isolated a new VHS-subtype /strain 23.75/ from brown trout /Salmo trutta/. The VHS viruses have been detected in Europe only.

The disease appears at a water temperature below 14°C. Highest mortality occurs at 8°C, the incubation period lasts 7-15 days and the mortality is usually high /50-80 %/.



	Titers of antibodies in serum carp No			Yield of virus in serum carp No		
	1	2	3	1	2	3
before treatment	<8*	<8	<8	-	-	-
28 days at 20°C	1024	256	12	-	-	-
14 days at 10°C	512	24	12	-	-	-
28 days at 10°C	128	48	12	-	-	-
14 days after challenge (10°C)	128	64	32	-	-	-

*Reciprocal of antiserum dilutions giving 50% plaque reduction.

Figure 5 Individual reaction of SVCV infected carp after lowering the water temperature from 20 to 10°C and after a challenge with 10^6 $TCID_{50}$ of SVCV

Table 4 Cross-neutralization between the three subtypes of VHS /after le Berre et al. 1977/

Antiserum	VHS-subtypes VHS-1	VHS-2	23.75
VHS-1	1000 ×	< 10	< 50
VHS-2	< 20	1000	< 10
23.75	300	20	1000

× (reciprocal of antiserum dilution giving 50% plaque count reduction)

Moribund fish appear dark and show exophthalmia. In the acute stage of infection haemorrhages in skeletal muscle, in swim bladder and in other internal organs are evident. In the chronic stage the pathological signs are usually very weak or absent.

Histologically, there are haemorrhages in vascular tissues and pathological changes can be seen in all internal organs. The kidney seems to be the primary target organ.

The virus is transmitted by carrier-fish, water, equipment and probably other vectors. Since the virus is lost from contaminated eggs by water flow /Jørgensen 1974/ a vertical transmission seems unlikely.

The only control measures are eradication of infected fish, disinfection of the entire hatchery and the use of water free of virus, as well as, disinfected eggs. Vaccines against VHS are being developed but at present there are no practicable methods of immunoprophylaxis available.

The virus is regularly isolated from several organs of infected fish showing the acute stage of infection. The agent is also present in ovarian fluid of infected brood stock fish. In carriers the virus is mostly not detectable. For seroepizootiol-

ogical surveys it should be stressed that the neutralization of VHS-virus using trout serum is complement dependent and it has been shown that the complement factor of trout is very labile /Dorson and Torchy 1979/.

Fish diseases with suspected viral etiology

Two contagious diseases occur in populations of cultured carp: a/ Epithelioma papillosum of carp; b/ Gill necrosis of carp.

The Epithelioma of carp is a localized epidermal hyperplasia of the skin of common carp /Cyprinus carpio/ and other Cyprinids. The causing agent has not been isolated but Schubert /1964/ demonstrated herpes virus-like particles in the hyperplastic epidermis of carp. Therefore, a viral aetiology of the disease is suspected. Infected carp show white verrucose lesions in the skin which can occur on every part of the body. The disease is not so interesting from econcmical points of view.

The gill necrosis of carp is a widespread disease in European carp cultures. Among several suspected aetiological factors /such as lack of vitamins, environmental factors, water quality, intoxications, bacteria/ a viral aetiology has been discussed also. Popkova and Schelkunov /1978/ isolated a cytopathogenic agent from carp afflicted with gill necrosis recently. The agent replicated in FHM-cells at 28°C producing cytopathogenic effects such as rounding up of cells and inclusion bodies in the nuclei. Electron microscopic studies revealed hexagonal particles 200--210 nm in diameter.

Further work is obviously required to clarify the aetiology of the disease.

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ISOLATION OF RHABDOVIRUS CARPIO FROM SHEATFISH (SILURUS GLANIS) FRY

N. FIJAN*, Z. MATAŠIN*, Z. JENEY**, J. OLÁH**, L.O. ZWILLENBERG***

*Department for Biology and Pathology of Fish and Bees, Veterinary Faculty, Zagreb, Yugoslavia
**Fisheries Research Institute, Szarvas, Hungary
***Laboratory for Biological Research, Bern, Switzerland

ABSTRACT

During rearing in a recirculation system at 22°C, a 90 per cent mortality of sheatfish fry could be observed between eight and 18 days of age. Samples of sick and dead fry having a hydropic and haemorrhagic syndrome were examined virologically. A rhabdovirus, which is serologically related to the causal agent of spring viraemia of carp /SVC/, was isolated on cell cultures. The role of the virus in mortality and the possible significance of the immunological immaturity of the fry for the outbreak of SVC or the SVC-like disease at 22°C, are discussed.

INTRODUCTION

At present, two acute viral diseases are known to cause mortality in fish species cultivated in European ponds: spring viraemia of carp /SVC/ and pike fry rhabdovirus disease /PFRD/.

SVC is an acute contagious disease caused by Rhabdovirus carpio /or SVC virus/, occurring in fingerling and adult common carp /Cyprinus carpio L./ during the spring with higher water temperature /Fijan et al. 1971, Fijan 1972/. The disease was previously regarded as a form of infectious dropsy of carp. Outbreaks of SVC and isolations of virus from carp were reported in several European countries /Fijan et al. 1971, Macura et al. 1973, Bachmann and Ahne 1974, Baudouy 1975, Rudikov et al. 1975, Kölbl 1975, Békési and Szabó 1977, Bucke and Finlay 1979/. It is suspected that SVC can cause mortality in several other fish species, but the virus isolation has been so far reported only

from naturally diseased bighead /Hypophthalmichthys nobilis Rich./ by Rudikov et al. /1975/, and from crucian carp /Carassius carassius L./ by Kölbl /1975.

PFRD has been causing severe losses in pike fry reared in Dutch hatcheries /de Kinkelin et al. 1973/. The rhabdovirus isolated by Ahne /1975/ from moribund grass carp /Ctenopharyngodon idella Val./, is serologically indistinguishable from pike fry rhabdovirus /Clerx et al. 1978/. There have been no other geographic and host records on PFRD, so far.

This paper is the first report on a sheatfish fry mortality associated with a virus. Here the isolation and properties of this rhabdovirus isolate will be described. It seems to be related to the R. carpio.

MATERIAL AND METHODS

Case history. About 130,000 sheatfish larvae 3-4 days' old were placed into the indoor fry rearing facilities supplied by the water from a recirculation system. This system was described by Csávás and Váradi /1980/. During the time of experiment common carp and sheatfish were the dominating species. Channel- and blue catfish, pike-perch, sterlet and European eel were also in the system but separately, and in lower number. The water was not treated for inactivation of fish pathogens. Water temperature was kept at 22-23°C.

Four days after the stocking of fry an increased mortality was observed. Losses continued during the next days. Diseased fish were dark and had a slight to moderately pronounced hydropic syndrome. Haemorrhages in fins, gills, muscles and internal organs were slight to moderate. Examinations for the presence of parasites and aggregations of myxobacteria on the external surfaces of fish were negative. Half-hour bath treatments with a combination of malachite green and formalin /treatment A/, were repeated three times daily on the second and third day after the onset of mortality, but they did not influence the course and outcome of the disease. Subsequent overnight bath treatments in a mixture of neomycin, oxytetracycline and copper sulphate /treatment B/ did not reduce the mortality either. Seven days

after onset of the disease the mortality rate decreased and about 6-8 per cent of fry survived.

Isolation and cultivation of virus. Six samples of fry were taken for virological examination on the sixth and seventh day after the onset of mortality. Samples 1 and 2 were taken before treatment B, samples 3 and 4 after the first course of treatment B and samples 5 and 6 after the second course of treatment B. Samples 1, 3 and 5 consisted of fry in initial stages of disease, sample 2 of moribund and samples 4 and 6 of freshly dead fry. In each sample there were 5-10 fish. Samples were frozen at -20°C.

For the examination, defrosted fry samples were minced and triturated in mortar by a pestle. The tissue was mixed with phosphate buffered saline at a ratio of 1:50. After sedimentation the supernatant was filtered through a Millipore HA filter /pore size 450 nm/.

Cell cultures were grown in Eagle's Minimum Essential Medium with Hank's salts, supplemented with 10 per cent fetal calf serum and antibiotics /100 i.u. of penicillin, 100 mcg of streptomycin and 0.2 mcg fungizone per ml/. All cultures used were 24 hours' old and nearly confluent.

For primary virus isolation, EPC /epithelioma papulosum cyprini/, FHM /fathead minnow, a cyprinid fish/, BB /brown bullhead, an ictalurid fish/ and RTG-2 /rainbow trout gonads/ cell cultures in test tubes were inoculated with serial tenfold dilutions of the filtrate from sample 2. The first three of the above mentioned cultures were incubated at 25°C and the RTG-2 cells at 20°C. Isolations and titrations from other samples were carried out in EPC cells grown at 25°C in microtitre plates. For other experiments, the virus was also grown in EPC cells on microtitre plates at 25°C.

Characterization of virus. Chloroform sensitivity of isolates was determined by the method of Feldman and Wang /1961/ and the influence of iododeoxyuridine /IUDR/ on the virus synthesis using Bader's method /1965/. Acid lability was tested with the method of Kelter et al. /1962/ using tris buffer at pH 3. In these tests, the SVC virus and the infectious pancreatic necrosis virus were used as controls.

Morphology of the isolate was examined by electron microscopy as described by Fijan et al. /1971/.

Virus neutralization /VN/ tests. Carp anti-SVC serum was obtained from fish weighing about 1500 g that were brought to the laboratory from a SVC-free farm. Their pre-immunization sera did not inactivate the SVC virus. Fish were kept at 15-17°C and inoculated intraperitoneally /i/p/ with about $1x10^6$ 50 per cent cell culture infectious doses /$CCID_{50}$/ of SVC virus strain S/30 /Fijan et al. 1971/. Two days after this inoculation, water temperature was gradually raised and maintained at about 23°C. Three weeks later carp were injected i/p with about $1x10^7$ $CCID_{50}$ of the same virus. Bleeding from the blood vessels in the hemal canal was performed seven days after the second inoculation. The inactivated serum neutralized 50 $CCID_{50}$ of virus, up to the dilution of 1:160. A serum dilution of 1:20 was used in VN tests described below.

Rabbit anti-SVC serum was obtained from B.J.Hill /Hill et al. 1975/. The endpoint dilution that neutralized 50 per cent of plaques was 1:5,600. For our VN tests this serum was diluted 1:200.

In addition to the isolate from sheatfish fry, the following viruses were grown in EPC cell cultures and used in VN tests: SVC virus strain S/30, SVC virus strain IBW 253/8 obtained from O. Kölbl, SVC virus "serotype II" supplied by W. Ahne and the PFR virus obtained from P. de Kinkelin.

Serial tenfold dilutions of each virus were mixed with equal volumes of normal carp and normal rabbit sera as well as with SVC antisera from these animals in plastic transfer plates. After incubation for 30 minutes at 20°C these mixtures were added to EPC cell cultures on microtitre plates. Inoculated cultures were incubated at 20°C and observed for cytopathogenic effect /CPE/. The rate of virus neutralization was expressed as neutralization index.

Optimal temperature for development of CPE. EPC cell cultures on five microtitre plates were inoculated with the same serial tenfold dilutions of the isolate and one plate of each was incubated at 10, 15, 20, 25 and 30°C, respectively. Plates were checked daily for development of CPE.

RESULTS

In EPC, FHM and BB cell cultures inoculated with filtrates from samples of sheatfish fry and incubated at 25°C, CPE was evident after two days. The titre of virus in 6 samples ranged between about $1x10^5$ and $1x10^8$ $CCID_{50}$ per 1 g of fish tissue. In the RTG-2 cell cultures inoculated with one of the samples and incubated at 20°C, CPE developed after five days.

All isolates were sensitive to ether. The isolate from sample 2 was chosen for further work. Its synthesis was not inhibited by IUDR. The isolate sensitive to pH 3, did not evoke a CPE at 30°C. At 25°C the CPE developed after 24 hours, at 20°C after 48 hours and at 15 and 10°C after five days.

Table 1 Results of virus-neutralization tests with Rhabdovirus carpio /SVC/ strains, pike fry rhabdovirus and the sheatfish isolate using carp and rabbit SVC antisera

Virus	Neutralization index	
	Carp antiserum	Rabbit antiserum
SVC S/30	3.2	3.0
IBW 253/8	3.1	1.70
SVC serotype II	3.0	1.75
PFR	1.0	1.0
Sheatfish isolate	2.5	1.75

Virions with a finger- or bullet-like shape could often be found in thin sections of infected EPC cells by electron microscopy. They were found both extracellularly and budding from cell membranes and vacuole membranes.

Results of VN tests are presented in Table 1. All the three SVC virus strains and the sheatfish isolate were neutralized similarly by the carp anti-SVC serum. With rabbit antiserum, the NI for strain S/30 was normal but for the other two SVC strains and for sheatfish isolate it was around 1.75. The PFR virus was neutralized only to a low extent by both antisera and recognized as a different virus.

DISCUSSION

Isolation of a virus and its high titres in samples of sick, moribund and dead sheatfish fry, the type of gross lesions in fish as well as the failure of several antiparasitic and antibacterial compounds to control the disease indicate that fry mortality in the described case was caused by viral disease. Experiments conducted so far on the pathogenicity of the isolated virus as well as the additional ones will have to be evaluated for definite judgement on the role of virus and of other factors in the particular mortality and in sheatfish culture in general.

The infection of sheatfish fry with the virus could take place either before or during the rearing in the recirculation system. The four-day interval between the placing of fry into this system and the onset of mortality, as well as the share of the undisinfected recirculated water supply with other fish species favour the latter hypothesis.

Morphology and the other determined properties of sheatfish isolate are typical for a rhabdovirus. Results of serological tests indicate a close relationship of this virus to Rhabdovirus carpio. A detailed serological study of this and other SVC isolates is needed before further subgrouping.

The outbreak of SVC or an SVC-like disease at a water temperature of 22°C is somewhat unusual. The cases of SVC so far diagnosed and reported in ponds occurred in springtime, mostly at water temperatures between 12 and 18°C and the mortality ceased with the increase of water temperature. Mortality may sometimes continue up to 22°C and this temperature was, therefore, mentioned by Fijan /1972/ as the upper thermal range of the disease. The single described spontaneous SVC outbreak in the laboratory took place at 10-15°C /Bucke and Finlay 1979/. In experimentally infected carp kept under various controlled temperature conditions Baudouy et al. /1980/ found the SVC mortality to be most severe at temperatures below 10-11°C; at 18°C and above the defence mechanisms were able to protect the fish from mortality. According to Ahne /1980/ and Fijan and Matasin /1980/ the infection of susceptible carp with SVC virus at 20°C does not result in mortality: at these and at higher tempera-

tures the defence mechanisms are able to restrict the virus replication and to eliminate it.

The reason for the reported SVC or SVC-like mortality in sheatfish fry kept at 22°C could be immunological immaturity. The ontogeny of the immune system in sheatfish has not been studied yet, but the available data on carp from van Loom et al. /1981/ allow the assumption that lymphoid organs of 8-18 day-old sheatfish fry /age of fish affected by mortality/ were not fully developed morphologically and functionally. In carp, the SVC infection of larvae and young fry results in high mortality both at 17°C and at 23°C /Fijan and Karovic 1977, unpublished/. This and other possible hypotheses on reasons for the virus-induced sheatfish fry mortality at 22°C will have to be explored experimentally.

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SKIN INFECTION OF THE SHEATFISH (SILURUS GLANIS L.) CAUSED BY A HERPES VIRUS

L. BÉKÉSI*, É. KOVÁCS-GAYER*, F. RÁTZ, O. TURKOVICS

*Central Veterinary Institute, Budapest, Hungary

ABSTRACT

Pox-like epidermal changes were found in a sheatfish population cultured in a net-cage system. The pathology was identical to that first described in 1970, but acidophilic inclusion bodies /Cowdry-A/ and several acidophilic autophage vacuoles were also found in the cytoplasm of the altered epithelial cells. In the nuclei and cytoplasm of these cells, herpes-like virus particles were detected by electron microscopy.

INTRODUCTION

Fish-pox is a well-known and frequently occurring lesion in the warm-water fish population of Europe. The disease and its histopathology have been described on many species other than carp. Concerning the etiology, the changes were earlier thought to be of metabolic origin, but using electron microscopy Schubert /1964/ reported a herpes virus in carp pox. His description has been confirmed in our country as well /Rátz et al. 1980/. The pathological changes with pox in the sheatfish were first reported by Lucky in 1970. Although he gave the exact histopathology of the lesions, the viral origin was only supposed.

MATERIALS AND METHODS

High mortalities accompanied by serious skin lesions were observed in a sheatfish population cultured in net-cages for two years. The disease appeared during the winter of the second year,

with the following gross pathology: greyish, slightly elastic coat that covered the skin surface in regions or formed mucous sheath mostly on the head. The affected animals showed a generally slimy appearance. Pathological changes could be seen on two thirds of the population and mortality was about 50% during the winter season /Figs. 1 and 2/.

Saprolegnia infection was often found on the gills and sometimes on the skin, too. The skin of the dead fish showed characteristic black lesions on the place of the original changes. No other change than liver distrophy was found in the organs. Samples from the affected skin were fixed in 10 % neutral formaldehyde, embedded in paraffin and stained with hemalauneosine /H.E./ and with PAS.

Tissue samples prepared for electron microscopy were first fixed in 5 % glutar-aldehyde buffered with Na-cacodylate followed by 1 % osmium-tetra-oxide. Durcupan was used for embedding. Ultrathin sections were stained with uranyl-acetate and lead--nitrate, and examined in a Philips 201-CS microscope.

RESULTS

Light microscopy revealed distinct thickening in the Malpighian layer of epidermis. In some of the epidermal cells the enlarged nucleus contained a central acidophilic inclusion body /Cowdry-A/. The nuclear membrane showed an irregular shape and was sometimes disintegrated. Acidophilic debris could also be seen in the pale cytoplasm. The thickened epidermis contained almost no mucus cells at all. The degenerated upper cell layers seemed to be gradually detaching from the deeper layers /Figs. 3 and 4/.

On electron micrographs nuclei of irregular form were found and chromatin density was higher around the nuclear membrane. Virus particles of 85-90 nm in diameter were scattered in the nuclei. The cytoplasm of these cells contained enveloped virus particles with a diameter of 145-160 nm. Autophage vacuoles were seen in the cytoplasm of degenerated epithelial cells /Figs. 5 and 6/.

Figure 1 Skin lesions on the head

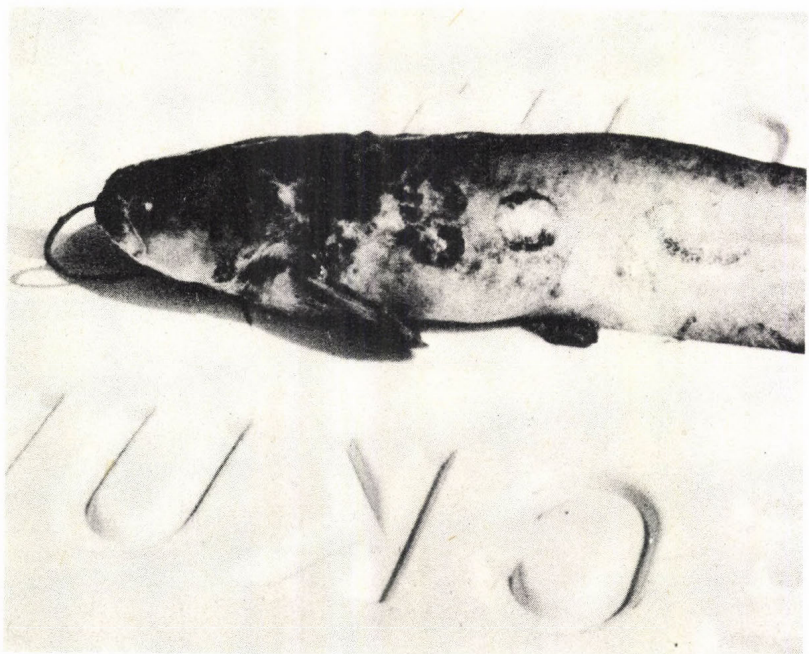

Figure 2 Post mortem changes of the skin

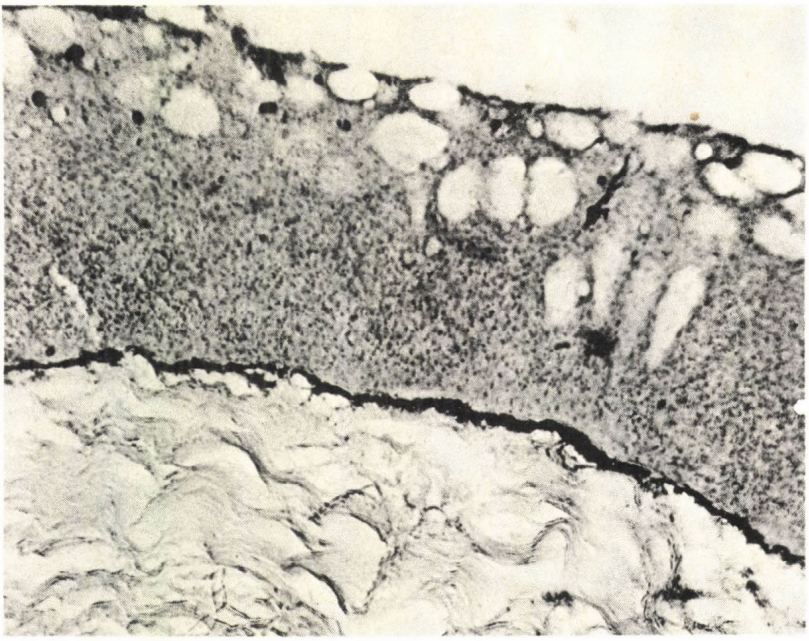

Figure 3 Hyperplasia of the Malpighian layer of epidermis with limited mucus cells /H.-E.,x 100/

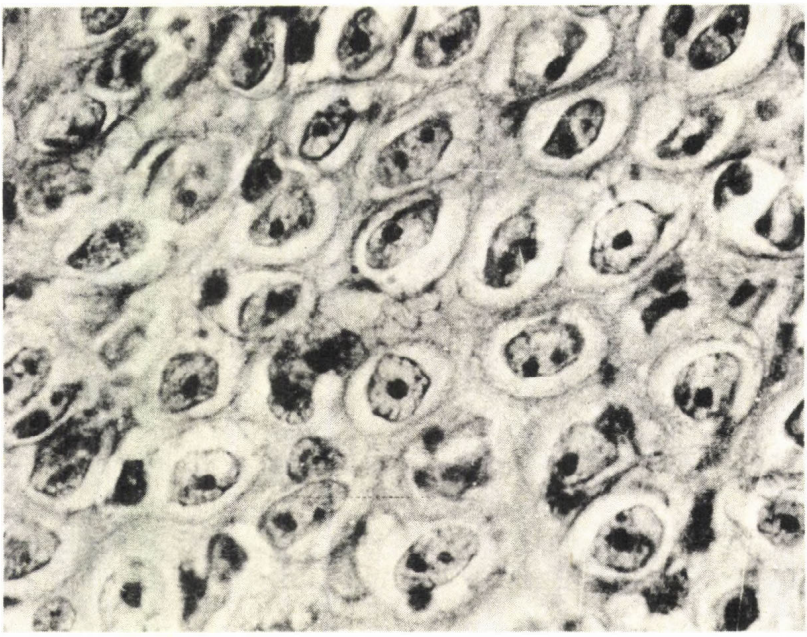

Figure 4 Inclusion bodies in the nuclei of the altered epithelial cells /H.-E., x 1,200/

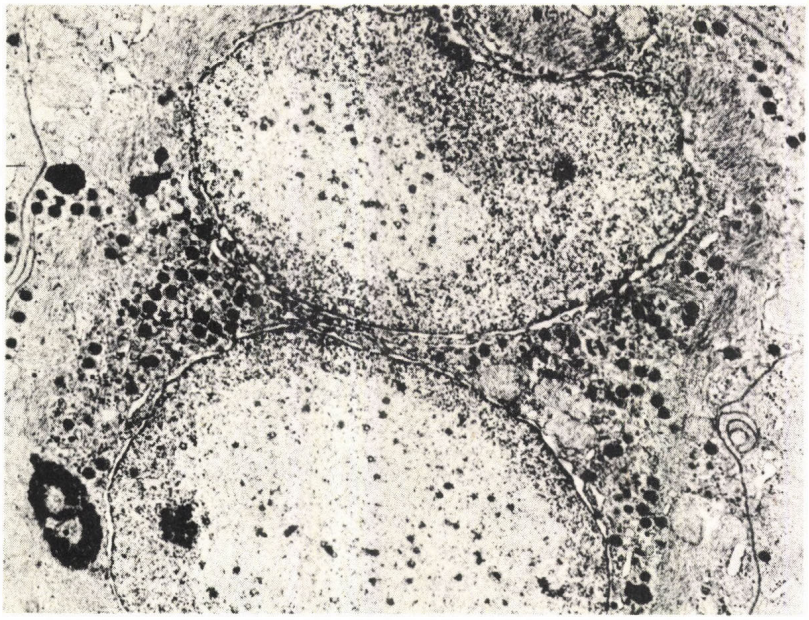

Figure 5 Virus replication in the nuclei and in the cytoplasm of epithelial cells /electron micrograph, x 10,000/

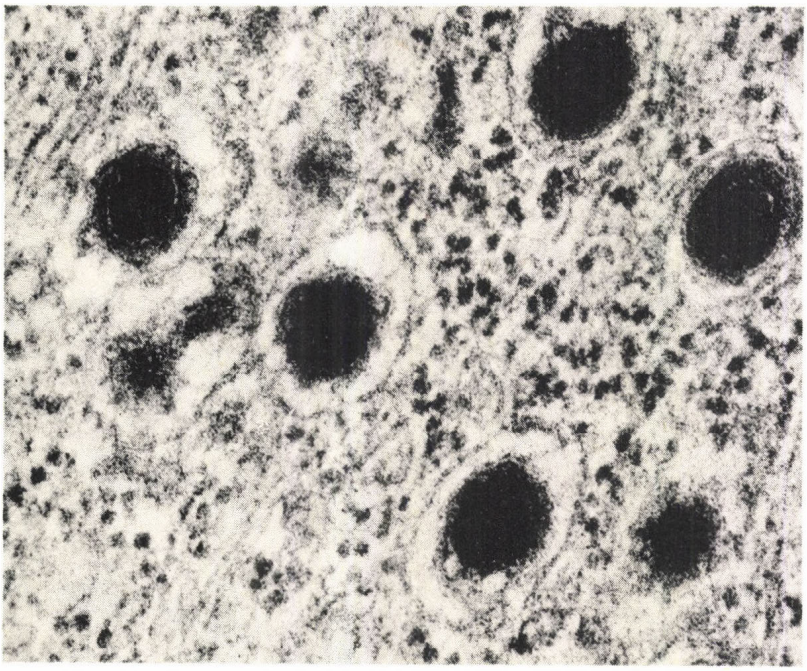

Figure 6 Virus particles with herpes morphology in the cytoplasm /electron micrograph, x 150,000/

DISCUSSION

The pox of sheatfish has been known in our country for a long time. But no mortality due to the disease has been observed yet. This prompted us to deal with the case in more details.

Its histopathology published by Lucky /1970/ was completed by us with the observation of acidophilic inclusion bodies as it was found in carp herpes infection. Further observation was, that the degeneration of the epithelial cells was faster and more profound in case of sheatfish. It was demonstrated by the many cell debris found in the cytoplasm of affected cells. This is also the reason why the upper epidermal layers of the sheatfish's skin desquamate more easily than observed in carp herpes. The sheatfish skin with different cellular structure is perhaps more predisposed to the virus infection.

Ultrastructural changes and the virus described above seem to be identical to those found in carp herpes. Attempts to propagate the virus have not been successful. We still regard this report as the first description of a herpes virus from sheatfish skin.

Further examinations are needed, however, to clear the role of this virus in the pathology of the disease. Our study should - first of all - help the histopathological diagnosis of the disease.

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RESULTS OF VIROLOGICAL STUDIES ON GILL NECROSIS

I.S. SHCHELKUNOV, T.I. SHCHELKUNOVA

All-Union Research Institute for Pond Fisheries, Moscow, Rybnoe, USSR

ABSTRACT

As a result of virological studies on carps diseased with gill necrosis, two viral strains have been isolated. The virions of both strains have a similar structure. Some physico-chemical properties of the virus have been determined, as well as the nature of the cytopathic effect in the cell culture and the peculiarities of virus reproduction in the cell. The virus is related to the family of Iridoviridae. The virus pathogenicity has been shown for the carp and relationship with the virus of carp gill necrosis is discussed.

INTRODUCTION

Nowadays gill necrosis is one of the most dangerous and widespread diseases of carp in the Soviet Union and in other countries. The etiology of the disease first described 20 years ago, has not been clarified yet. For a long time, different diseases with similar clinical symptoms were taker for gill necrosis /Bohl 1978/. By the middle of the 70ies, two different viewpoints came about on the nature of the disease.

According to Belorussian scientists the disease is of virus origin. Virus could be isolated from diseased carps and the symptoms could be induced with experimental infection /Linnik et al. 1972, Mamish 1975, Galavhov and Ekelchik 1978/.

In the opinion of other authors the disease is not infectious. Musselius and Chernishev /1974/ could not induce it in healthy fish either by keeping themtogether with diseased fish or by

infective material. They do not consider the disease infectious with the presumption that it is caused by the impairment of hydrochemical parameters due to the over-accumulation of organic matters in the water /Musselius et al. 1974/.

Schreckenbach et al. /1975/ found that the disease is caused by exogenous ammonia through auto- or intoxication coming about at high ammonium-ion content and increased pH value of the water.

Experimentally the disease could be induced by adding certain materials, thus creating a hydrochemical model situation leading to the formation of the disease /Ivanov et al. 1976/.

Earlier we reported on the isolation of virus from fish diseased in gill necrosis and described some morphological and physico-chemical properties /Popkova and Shchelkunov 1978, Shchelkunov and Shchelkunova 1979/.

In the present paper we have summarized the results of our virological studies.

MATERIAL AND METHODS

The applied cell lines and primary tissue cultures

For isolation and passage of the virus- tripsinated primary tissue culture of carp gonad, FHM /Pimephales promelas/ and EPC /Cyprinus carpio L./ cell lines were used /Osadchaya 1969/. FHM and EPC cell lines were cultured in Eagle's MEM medium containing 10 % fetal bovine serum.

Collecting and processing of the samples

Samples were collected from carps of the following ages: one-, two- and three-summer old carps and spawners. The clinical pattern of the diseased fish examined was the following: gills were edematous and coated with mucus, lamellae had mosaic-like pattern, discoloured. In some cases anemia of the gills could also be observed. Tissues of liver, kidney and spleen, respectively, of 5-10 fish were mixed for further examination. Samples from each spawner was later mixed by 2-3 items. Gill tissues were collected as described above but handled separately from the parenchymal organs. Tissue samples were then put into sterile test-tubes containing either Eagle MEM medium or Hank solution and placed into thermos with ice and transferred to the labora-

tory. The processing of the samples was carried out according to generally accepted methods /Wolf 1970, Hill 1976/.

Inoculation of cell cultures and passage of cytopathogenic agents

0.1-0.2 ml of bacterium-free samples was put into the cell culture containing tubes. In each case 3-4 cell cultures were infected, after washing the monolayer twice with serum-free medium. After 1/2-1 hr absorption time at room temperature, medium containing 1-5 % serum was added to each tube. The incoulated cell cultures were incubated at 22 and 28°C, respectively. If the cells of the monolayer were affected in 75-100 %, the samples were frozen at a temperature of -20°C and after thawing new cultures were inoculated with this material. The same procedure was performed with the samples showing a weaker cytopathogenic effect or did not affect the monolayer in 7-10 days at all. If not cytopathogenic effect could be observed after 3-4 consecutive passages the material was regarded virus-free. In most cases only one of the above mentioned cell lines and primary tissue culture was used for isolation; occasionally two of them were applied.

Virus titre

Virus titre was determined from ten-fold dilution of the virus-containing material and then, cell cultures were infected with the dilutions. TCD_{50} was determined according to the methods of Read and Mench.

Luminescence microscopy

FHM cell line was cultured on coverglass and inoculated. After the appearance of cytopathogenic effects the cell culture was rinsed with Hank solution, and fixed in ethyl alcohol for 5 min, dried and stained with one drop of acridine orange, then covered with glass and viewed under the luminescence microscope. Acridine-orange solution was prepared with phosphate buffer /pH 7.4/ in a concentration of 1:50,000.

Heat sensitivity

The virus containing culturing medium /together with cell debris/ was incubated at different temperatures: -20°C, +4°C, +27°C, +56°C. The inactivation of the virus was judged on the basis of the titre-decrease.

Ether, chloroform and acid sensitivity of the virus

Virus sensitivity was tested according to accepted methods /Starke 1970/ in 0.5 % ether and 5 % chloroform. To determine acid sensitivity /pH 3.0/ virus containing medium was distributed into two parts. To 1 ml of the first part 5 ml buffer /pH 3/ was added /buffer: 4.1 % 0.2 M Na_2HPO_4 + 15.89 % ml of 0.1 M citric acid/. After incubation at room temperature for 30 min, the mixture was neutralized with isotonic Tris-HCl buffer, pH was adjusted to 7.5. While with the second part, which served as a control, the same procedure was performed with the exception that instead of 5 ml buffer of pH 3, Eagle MEM medium was added. The degree of inactivation was estimated on the basis of the decreased titre of the treated virus.

Electron microscopy

For electron microscopic investigations the apical and mesial parts of gill lamellae of carp with chronic gill necrosis was used. The gill sections were fixed in 4 % glutar dialdehyde and in 1 % OsO_4, dehydrated in acetone then embedded in epon-araldite. Differential staining of the ultra thin section was made in saturated water solution of uranyl acetate. The virus infected FHM samples were prepared in similar way. For negative differential staining only debris free cultures were used which showed an expressed cytopathogenic effect. The virus was adsorbed onto carbon coated microsieves and stained with 3 % solution of sodium phosphotungstate /pH 6.8-7.0/. The samples were examined in JEM--100B electron microscope.

Experimental infection of healthy carps

To establish the pathogenicity of the isolated virus, 8 experiments were designed in which healthy carps were infected with virus containing culturing medium or with tissue samples taken from experimentally infected carps. Virus infected FHM or EPC cell lines were used after subsequent freezing and thawing. The control group was inoculated with virus-free cell culture medium.

The experiments were carried out with one- /average weight of 18-20 g/ and two-summer-old carps /230-260 g average weight/ from fish farms free of gill necrosis. The fish were infected

either by injuring gill or by intraperitoneal injection. 5-10 fish were infected with both methods. The dose of virus in the culturing medium during infection was 5.10^{-3}-$2.5.10^{5}$ TCID per fish. Fish were kept at a water temperature of 18-23°C without feeding. The duration of each experiment was at least 20 days.

RESULTS

Passage of the samples

Altogether 61 samples were collected from 8 fish farms of the Soviet Union, where gill necrosis could be detected. From these samples two virus strains were isolated on FHM cell culture at 28°C.

One of the virus strains, marked 1LZ was isolated from carp breeder and the other one, 4BZ from a two-summer-old carp. Both viruses were isolated from gills.

The strain 1LZ grew well on FHM cell line culture and damaged the monolayer culture during the first four passages in 1-2 days. Its pathogenicity, however, dramatically decreased further. In the tenth passage no cytopathogenic effect could be observed at all. The virus presumably lost its pathogenicity for FHM cell line.

The 4BZ strain showed increasing pathogenic tendency. While at the beginning, it damaged the monolayer in 7-9 days, this time decreased to 1-2 days in the subsequent passages. Up to the present, this strain has been subject to more than 30 passages on primary cell cultures as well as on cell lines. Every experiment presented in this paper was performed with the latter strain.

Cytopathogenic effect of virus

The virus-infected cells were stained with Romanovsky-Giemsa method and examined under the light and luminescence microscope. It was found that first of all the nucleus changed in infected cells by taking up an irregular shape. During luminescence microscopy, we observed green coloured elements in the nuclei referring to the presence of DNA.

During light microscopy increased basophilia of the nucleus and in it one or more intensively staining inclusion bodies could be observed on the onset of the infection process /Popkova and Shchelkunov 1978/. The increased basophilia of the nuc-

leus was followed by increase of the cytoplasmic basophilia, too. The cells lost their cytoplasmic protuberances, gradually became round then detached from the culturing glass. During a more elongated formation of the infection process, transparent, spheroid cells of increased size could be seen. The virus titre was measured where at least 75 % of the monolayer culture was damaged. In such culturing medium the virus titre measured was 10^{4}-10^{5} $TCID_{50}$ ml^{-1} after one subsequent freezing-thawing.

Stability of the virus at different temperatures

We found the virus relatively heat-stable. At a temperature of -20°C it retained its properties for 3.5 years /i.e. the duration of the study/. Owing to storage at 4°C, the virus titre decreased to a value of $5.10^{+0.5}$ $TCID_{50}$ ml^{-1}. +27°C temperature was applied to study the speed of heat-inactivation of the virus during the incubation when infecting the different cell lines. We found that at this temperature the inactivation was relatively elongated: the decrease of virus titre was 68.4 % and 90 %, in five and ten days, respectively. 56°C heat stress for 30 min, partly, and for one hour completely inactivated the virus.

Ether, chloroform and pH sensitivity

The virus was resistant to ether, at the same time chloroform completely inactivated it. A buffer of pH 3 decreased its titre by 99 %.

Electron microscopy

Virions of both strains were of identical structure. Their form is of an icozaeder and 190-210 µm in diameter. The capsid wall consists of two layers which can be seen very well in preparations negatively contrasted /Figs. 1, 2/. Every layer consists of morphologically independent units, so called capsomeres. The quantity of these capsomeres could not be determined either in inactivated virus preparations or in preparations purified from "ballast" proteins with 0.5 % chloroform. During the investigation of purified preparations, we found that the capsomeres are spheric with a diameter of 6.5 µm /Fig. 3/, and are concentrated in the viroplasts of the cell-cytoplasm of the mature virion /Fig. 4/. The virus is not coated.

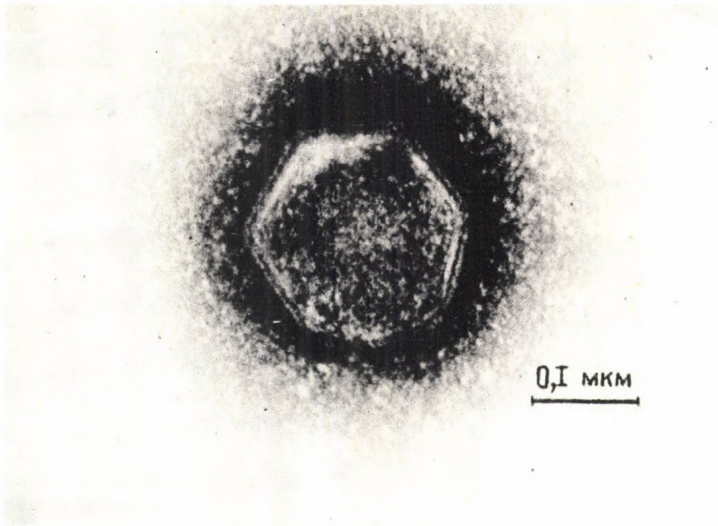

Figure 1 Negatively contrasted preparation of the virus. 1LZ strain

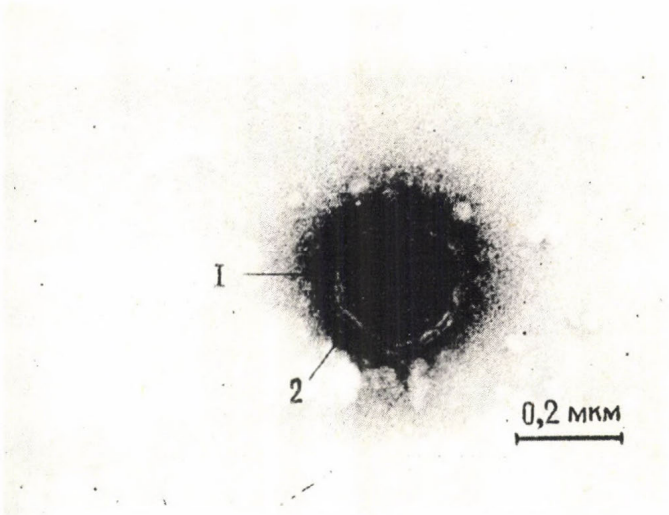

Figure 2 Negatively contrasted preparations of the virus. 4BZ strain. 1 - outer, and 2 - inner layer of the capsid wall

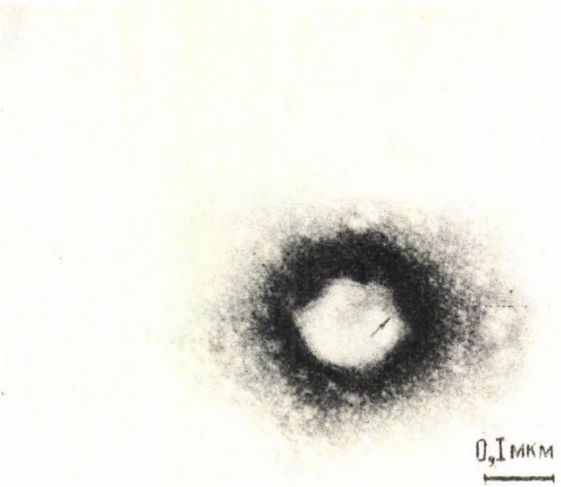

Figure 3 Preparation of the virus purified with 0.5 % chloroform. 4BZ strain /negative contrasting/. The arrow shows the part of the capsid where capsomeres can be seen

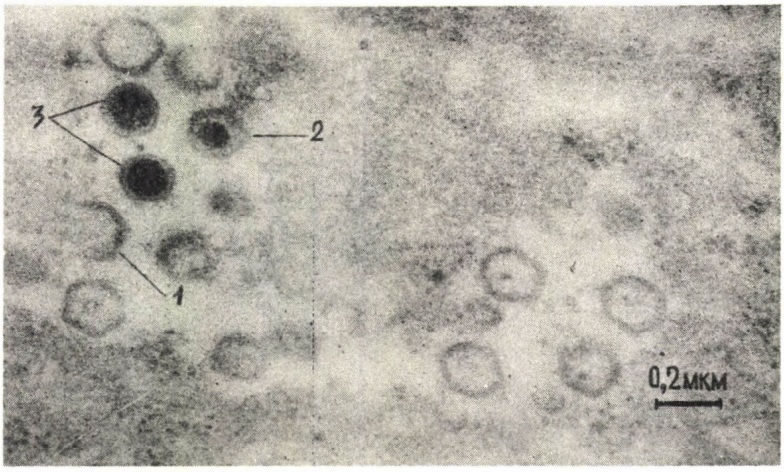

Figure 4 Multiplication of the virus in FHM cell culture. 4BZ strain. 1 - capsid of the virion in the stage of aggregation; 2 - virion in the stage of filling up with nucleic acid; 3 - mature virion

50 specimens of infected gill tissue were investigated. Beside normal ones, cells of different stages of degeneration could also be found. No virus-like agents could be observed.

The pathogenicity of virus to carp

There was only one out of 8 experimental infections, when clinical symptoms characteristic for acute form of the disease could be induced. This experiment was carried out with intraperitoneal injection of 2-summer-old carps. Necrotic symptoms characteristic on gill necrosis could be observed on 7 out of 8 fish /dropsy and mosaic pattern of the gill, white discoloration, and necrotic initiation at the tip of the lamellae/. The liver of fish was grey-yellow, loam coloured. From this material the virus could be re-isolated. Another group /8 carps/ was infected with these samples and the symptoms registered /3 fish died after 18 days/. No virus could be re-isolated from these samples.

Unsuccessful virus isolated of the latter seemed to indicate that the virus is not a pathogen for carp and cannot propagate in it, thus the virus re-isolated was the same after it had been administered to the organism. To confirm this presumption we designed experiments for quantitative determination of the virus in organs of experimentally infected fish. 20 two-summer-old carps were injected intraperitoneally with a dose of $7.5.10^{4.5}$ $TCID_{50}$. Four fish each were investigated 4, 9, 14, 21 and 37 days after infection and the quantity of virus determined in the gill and in the sample mixture of parenchymal organs.

No change could be observed in the gill during the time of observation /37 days/. The livers of 15 fish were grey-yellow, loam coloured and spleens swollen in certain cases. No alteration could be detected in organs of fish investigated on the 4th day, and nor virus could be isolated from the gill of infected fish.

Figure 5 shows the change of virus measured in the parenchymal organs and calculated for one fish. It should be noted, however, that the cytopathogenic effect could be experienced only at the titre-determination at the second-third passage.

As it turns out from Figure 5, the virus titre measured on the 9th day after infection was $2.5.10^{5}$ TCID, which is approximatelly in line with the administered quantity.

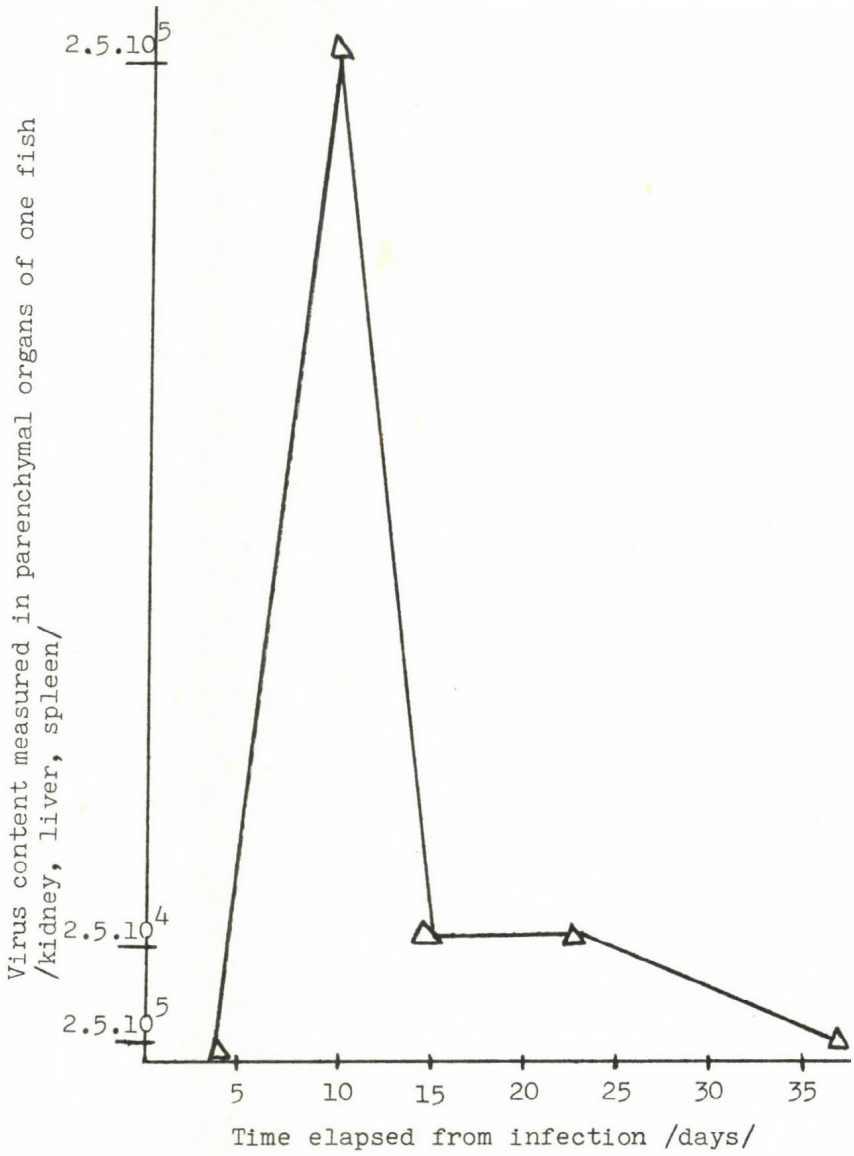

Figure 5 Dynamics of virus content measured in parenchymal organs of experimentally infected fish

Considering that the virus can be found in other organs of fish as well and that due to immunological defence mechanism of fish its quantity decreases, it can be concluded that the virus propagates in the fish.

DISCUSSION

Virus could be seldom isolated from carps diseased with gill necrosis /2 cases out of 61/. It can be explained by the fact that the FHM and EPC cell lines used for isolation were not susceptible enough in case of the given virus. This should be dealt with in further.

The structure of virion, the cytopathogenic effect and virus multiplication were described earlier /Musselius et al. 1974/. These data correspond to those published earlier /Kasarjeva et al. 1978, Szikalo et al. 1979/. It is also possible that the two viruses are identical or at least are very close to each other, but this should be tested with serological methods.

Viruses isolated by Mamis T.I., Solovnyov L.N., and us, are different in respect of certain physico-chemical properties, which, however, can be explained by the errors of the methods applied. On the basis of structure, size of virions, the way of virus multiplication in the cell as well as of physico-chemical properties found, we can presume that they belong to the strain of Iridoviridae. Beside viruses causing necrosis of lymphocytes and erythrocytes, presumably this is the third member of this virus strain isolated from fish. This, however, needs further investigation to be confirmed. The character of DNA containing inclusion bodies which are possibly the result of nucleus chromatin margination and can be observed with luminescence microscopy, is not known.

As it has been recently confirmed with experiments performed with FV-3 viruses of frogs, a certain part of the virus DNA is synthesized in the nuclei of the cells /Goorke et al. 1978/. Experimental infections have proved that though its virulence is low, the 4BZ virus strain is a pathogen for carp. It is also possible that pathogenicity decreased because of the subsequent passages. The virulence of the strain isolated by Belorussian scientists remained high even after passage on cell cultures.

Results obtained so far do not clearly prove that the isolated virus is the pathogen of gill necrosis of carp, so further work is necessary to elucidate the problem.

SUMMARY

Two virus strains were isolated from carps diseased in gill necrosis, and cultured on FHM cell line.

The structures of both virus strains were identical. We classified the strain as Iridoviridae. The virus is the pathogen for carp, its pathogenicity, however, is low. It can induce a disease of carp which proceeds without manifestation of clinical symptoms and liver is damaged first of all.

The structure of virion, the character of the cytopathogen effect in cell culture is identical to data described with gill necrosis of carp, which is not sufficient, however, for confirming the identity of the two viruses.

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BACTERIAL DISEASES

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OCCURRENCE OF PASTEURELLA AND STREPTOCOCCUS IN SHEATFISH (SILURUS GLANIS L.) AND COMMON CARP (CYPRINUS CARPIO L.)

J. FARKAS, J. OLÁH

Fisheries Research Institute, Szarvas, Hungary

ABSTRACT

The present report describes the occurrence of two relatively rare bacteria. The Pasteurella-like bacterium was isolated from 2-year-old cage-reared sheatfish. The disease appeared in summer when the water temperature was higher than 20°C. The bacterium causes septicaemia and high mortality of fish. The bacterium isolated from spleen and liver was similar to Pasteurella piscicida isolated by Jannsen and Surgalla /1968/.

Streptococci belonging to serotype D were first isolated from carp gills attacked by so-called "gill necrosis". This bacterium was regarded as the causative agent of the disease. Artificial infections were carried out and the characteristic symptoms of the disease appeared occasionally after the pathogen was smeared on the gill. The bacterium could be isolated again from the parenchymal organs and gills. During the subsequent experiments, it became obvious that the gill changes examined /so-called gill necrosis/ were caused by myxobacteria and it was a kind of bacterial gill disease. The Streptococci are incidental bacteria, the role of which has not yet been clarified in gill necrosis. They were present on the gills damaged by myxobacterial gill disease. Investigating 500, mostly healthy fish the bacteria were present on the symptom-free gills. The rate of occurrence was 0-20 % depending on water temperature. For this reason, the Streptococcus isolated /like other fish pathogen bacteria/ may be considered a member of normal gill microflora. The present paper describes the physiological-biochemical properties of these bacteria.

INTRODUCTION

Pasteurella and Streptococcus are rather rare and little known pathogens of fish. Snieszko and co-workers reported first an epizootic disease of Roccus americanus caused by Pasteurella in 1964. The strain /named Pasteurella piscicida/ isolated during this epizootic was first described by Jannsen and Surgalla in 1968. Similar bacterium was isolated later in Japan by Kubata et al. /1980/. In 1976, Hostein and Bullock described it in Europe, as a Pasteurella-like bacterium pathogen of trout and salmon, though according to Bullock the bacterium isolated was rather an atypical Aeromonas salmonicida. In each case, the diagnosis was septicaemia.

Scanty data are available on the fish pathogenicity of Streptococci. In each case described so far, the pathogen was isolated from parenchymal organs. Up to now, it has been isolated from diseased rainbow trout by Boomker et al. /1979/, from yellowtail by Kusuda et al. /1976/ and from eel /Kusuda and Komatsu, 1978/ mostly from kidney. Most of the Streptococci isolated in Japan, the United States and in South Africa were of D serotype and were similar to Streptococcus faecalis or faecium, with the exception of those found by Kusuda et al. /1978/.

In the present communication, we shall deal with the occurrence of these two bacteria rare in Hungary. This Pasteurella-like bacterium was first isolated from sheatfish /Silurus glanis L./ in the summer of 1977. Streptococci were isolated in great number and frequently during bacteriological examination of the so-called "gill necrosis" of carp /Cyprinus carpio L./.

MATERIAL AND METHODS

Pasteurella was isolated on nutrient agar plates from 6 diseased or freshly dead sheatfish.

Streptococcus taken from gills of healthy and diseased carps were further cultured in thioglycolate broth complemented with Na-azid. During the first series of experiments 25-30 carps with "gill necrosis" were examined. Later, to estimate the occurrence of the bacterium, we took samples from the gills of 500 mostly healthy carps on five occasions. The presence of Streptococcus in thioglycolate broth was tested by Gram staining.

17 Pasteurella and 14 Streptococcus strains were tested according to Hodking and Collee /1971/. Serological investigation of the strains was performed at the Veterinary Research Institute of the Hungarian Academy of Sciences /Budapest/. Antibiotic sensitivity test was made with the antibiotic discs - Resistest - of "HUMAN" Pharmaceutical Factory, Budapest.

RESULTS

1. Pasteurella

The process and symptoms of the disease caused by Pasteurella were the following. Sheatfish were cultured in net cages of a horse-shoe lake. During the outbreak of the disease the water temperature was higher than 20°C. The movements of the infected animals became slow and mucus developed on the skin. The skin colour became silverish. The abdomen was swollen and filled with light-yellow fluid. The guts and stomach were also swollen and white spots could be found in the kidney. Haemorrhagic spots were on the body especially on the abdominal part and at the base of fins. Mortality was significant.

The bacteria isolated from kidney, liver, spleen and blood were further cultured on nutrient agar plate. During 48 hours small colonies of 0.5-1.8 mm in diameter developed if the temperature of incubation was 30°C. The white coloured, transparent colonies were round, slightly convex with smooth edge. The physiological-biochemical properties of our strains are compared with those of Jannsen and Surgalla's strains /1968/ /Table 1/.

The bacteria are small Gram negative rods - so called coccobacilli. Their size is 0.5x1.5 μ. Staining has bipolar characteristics. In older broth, they are definitely pleiomorf and make the media turbid. All the properties are identical with those described by Jannsen and Surgalla /1968/ with the exception of gas formation from galactose and mannose not characteristic for Pasteurella.

The strains are properly or moderately sensitive to the majority of antibiotics, so there is a wide range of medicaments at our disposal if necessary /Table 2/. Ampicillin was suggested by Kusuda and Inoue /1976/, while Bullock chose sulphonamide for prevention and therapy.

Table 1 Characteristics of Pasteurella-like bacteria isolated from sheatfish, compared with Pasteurella piscicida isolated from white perch

	Strains from white perch, Jannsen and Surgalla /1968/	Strains from sheatfish
/!/ Gram's staining, motility	-	-
/!/ Growth at 37°C	-	-
/!/ C. oxidase, catalase	+	+
H_2S	-	-
NO_3 red.	-	-
Urease, indole	-	-
V-P test	-	-
Gelatinase	-	-
Methyl red	+	+
/!/ Glucose fermentation		+
Acid and gas /G/ from glucose	+	+
fructose	+	± /10/
sucrose	+	± /8/
maltose	+	± /14/
mannose	+	+ G
galactose	+	+ G
trehalose	-	-
arabinose	-	-
raffinose	-	-
dulcitol, xylose	-	-
inositol, cellobiose	-	-
mannitol, lactose	-	-
salicin, sorbitol	-	-
inulin, dextrin, adonitol	-	-

/ / number of positive reactions from 17 strains
According to key characters marked /!/, the isolates may be a kind of Aeromonas salmonicida.

Table 2 Sensitivity of Pasteurella to antibacterial agents

Sensitive or moderately sensitive	Resistant
Nitrofurantoin /300/	Methicillin /20/
Chloramphenicol /30/	Polymyxin B /15/
Penicillin /31.U/	Superseptyl /400/
Streptomycin /30/	Novobiocin /30/
Oxytetracycline /30/	Lincomycin /10/
Carbenicillin /50/	Oxacillin /10/
Gentamycin /20/	Oleandomycin /30/
Chlortetracycline /30/	Erythromycin /10/
Kanamycin /30/	Vancomycin /50/
Ampicillin /20/	Spiramycin /30/
Neomycin /100/	Pristinamycin /10/
Paramomycin /50/	
Cephalosporin /10/	
Tetracycline /30/	

/ / µg effective agent/disc

Artificial infections were performed to define pathogenicity. The results of experiments in aquaria were not unequivocal. We presumed that pathogenicity depends on predisposing environmental factors.

Since no serodiagnostical tests have been carried out so far - we defined them as "Pasteurella-like" bacteria. According to some key characters the isolates may be also an atypical Aeromonas salmonicida.

2. Streptococcus

An assessment of gill necrosis of carps was the first occasion when we detected Streptococcus. 25-30 diseased carp were examined when Streptococcus was found in 80-90 % of the cases. The bacteria were mainly on the gill but we could isolate them from parenchymal organs as well, especially after artificial infections.

To estimate the frequency of its occurrence, gills of 100 fish were examined on 5 occasions, and Streptococcus found on gills of apparently healthy fish in 0-22 %. This 22 % was found

Table 3 Characteristics of streptococci from carp gills

Gram's stain	+	Oxidase	-
Motility	-	Litmus milk	acid
Hydrolysis of hippurate	-	H-L test	ferm
Growth at pH 9.6	±	Acid from glucose	+
6.5 % NaCl	±	glycerol	-
45°C	+	cellobiose	+ /13/
10°C	+	inositol	+ /11/
Growth in meth. blue 0.3 % milk	+	mannose	+
Hydrolysis of tyrosine	+	arabinose	± /9/
arginine	+	sucrose	+
starch	-	mannitol	+ /12/
esculine	+	xylose	+ /12/
M-R test	+	galactose	± /8/
V-P test	-	dulcitol	-
Indole	-	lactose	± /9/
Gelatinase	-	fructose	± /9/
Urease	-	raffinose	± /7/
NO_3 red.	-	trehalose	+ /11/
H_2S	-	sorbitol	- /3/
Utilisation of citrate	-	salicin	± /9/
malonate	-	adonitol	-
Hydrolysis of tween-80	-	rhamnose	± /5/
trybutyrin	-	starch	+ /11/
2,3 but.diol.	-	melibiose	- /3/

/ / number of positive reactions from 14 strains

in July, when the temperature of water was higher than 20°C. According to these data, Streptococcus can be regarded a member of gill's normal microflora.

At any rate, it is certain that it can be isolated in 88--100 % of myxobacterial gill diseases as a concomitant bacterium. Similarly to myxobacterium it disappears in the recovery stage of the disease. The results of physiological-biochemical investigations of 14 Streptococcus strains are shown in Table 3. The bacteria are non-motile, Gram positive, chain-forming cocci. They ferment glucose, form acid from carbohydrates but never produce gas. They grow at 10 and 45°C. They decarboxylate tyrosine, arginine and hydrolase esculine. Methyl-red test is positive. The growth of bacteria at pH 9.6 and in the presence of 6.5 % NaCl is unstable. On the basis of serotypization, the strains examined belong to D serotype and, collating these with other properties, the bacterium must be close to Streptococcus faecalis and faecium species.

As in the first stage of our investigations we regarded this bacterium as a co-pathogen of gill necrosis - we performed experimental infections.

In experiments we smeared the bacterium on the gill. In few cases, symptoms resembling gill necrosis appeared but they were not typical. In other experiments carp fry were kept in bath /solution/ containing Streptococcus for a short time and then for 3 months under permanent control. 3 carps out of 17 survived the experiment.

In short, the disease caused by Pasteurella is rare in Hungary, but it is a potential pathogen - the epidemic attack of which might be very dangerous and should be counted on. The bacteria perhaps may be atypical Aeromonas salmonicida. The occurrence of Streptococcus is very frequent, especially on the gills attacked by Myxobacteria. Though its role has not been clarified yet - it is worth dealing with it.

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OCCURRENCE, EXPERIMENTAL INFECTION AND TREATMENT OF MYXOBACTERIAL GILL DISEASE OF CARP

J. FARKAS, J. OLÁH

Fisheries Research Institute, Szarvas, Hungary

ABSTRACT

Controversial viewpoints came about on the aetiology of gill necrosis. According to East German experts this disease is caused or initiated by ammonia but there is strong evidence on the bacterial or even viral nature of the disease. The present paper describes the Myxobacterial origin of the gill disease.

Both gills of 100 carps of different age groups were examined on 20 occasions in different pond management systems. On the basis of these results, the frequency of Myxobacterial gill disease was 0-50 % giving an average of 5-10 %.

We found that the appearance of Myxobacteria was preceded by stress conditions /i.e. high temperature and increased ammonia concentration of water, high pH value/. Number of bacteria were isolated both from healthy and diseased gills. From these bacteria, Aeromonads, a Streptococcus of D serotype and a Myxobacterium could be regarded pathogen. On the basis of experimental infections it turned out, that Myxobacterium was responsible for the typical symptoms, though bacterium could not always be detected on gills infected in the ponds. Physiological, biochemical characteristics and antibioticum resistance of 8 Myxobacterium strains were determined.

The following artificial infections were performed to reproduce the disease: /a/ smearing Myxobacteria on the gill, /b/ bathing carps in water containing Myxobacteria, /c/ bathing carps with injured gills in water containing Myxobacteria. In case /a/ the symptoms always appeared on the gill but low water temperature was a limiting factor. In cases /b/ and /c/ the infection

depended on the temperature of water and duration of bathing and conditions of fish. The Myxobacterial disease of gill could be prevented with chloramphenicol, oxytetracyclin and an antibiotic "X" either by injecting or feeding these drugs.

INTRODUCTION

During the past few years serious difficulties have been caused by the disease named "gill necrosis", though its real causes are not known yet. According to Schreckenbach and Spangenberg /1978/ the necrosis is caused by high ammonia content of water and blood. Myxobacterial infections of the gill should be separated from gill necrosis. There is also an opinion that gill necrosis is caused by viruses. The present paper shows that the complex of symptoms - called gill necrosis - which is initiated by environmental factors, often turns into Myxobacterial gill disease. Artificial infections proved that Myxobacteria can exert the symptoms on carp gills.

Bootsma stated /1974/ that the so-called gill necrosis of carp is of Myxobacterial origin. This opinion was confirmed by Bullock's /1972/ experimental infection, who managed to produce the same symptoms in trout. In his experiments, ammonia caused only microscopical damages in gill tissues, while the disease became manifest when Myxobacteria, Aeromonads and Pseudomonas were used as infecting agents. Plumb et al. /1976/, Walters and Plumb /1980/ also regarded ammonia an initiating agent of disease caused by Aeromonas infection in channel catfish.

In our work three kinds of bacteria could be regarded as pathogenic. These were the following: Aeromonads, a Streptococcus of D serotype and Myxobacteria. Since during experimental infections it turned out that Myxobacterium was responsible for the characteristic symptoms, we will deal only with this bacterium.

MATERIAL AND METHODS

Our first task was to assess the occurrence of Myxobacterial gill diseases in several carp populations. Both gills of 100 carps were examined on 20 occasions during autumn fishing and some other times. The presence of Myxobacterium was detected microscopically.

Bacteria were isolated from body surface, gills, kidney, liver, blood, gut and brain of both healthy and diseased carps. Appreciable results were obtained only from gills.

The experimental infections were performed on fingerling and one-year old carp with Aeromonas hydrophila, Streptococcus and Myxobacterium. Unambigous results were obtained only with infections with Myxobacteria. The ways of infection were the following:

- By smearing bacteria on gills with bacterial loop. Bacteria were cultured on agar medium. Gills were injured during smearing.

- By bathing carps for some hours in water containing bacteria. Bacteria were added to the water in the form of 24-hour--old Myxobacterial broth culture with a dilution range of 1:20 - 1:100.

- By a combined treatment; Injuring and bathing. The procedure was similar to the foregoing. Before bathing, both gills were injured and then the fish were kept in bathing solution for 5 minutes, 1 hour and 4 hours, respectively.

- By heat-treatment. The water where the fish were kept was heated from 12-13°C to 20-22°C. This temperature was then maintained for one week. At the water temperature indicated, infection developed spontaneously.

To work out methods for prevention with antibiotics, smearing on gill or water heating technique were employed. Infected fish were injected or fed with antibiotics. Antibiotics of 20 mg/kg fish were used in case of chloramphenicol and oxytetracycline, and 10 mg/kg fish from the antibiotic named here "X".

RESULTS

Data representing the occurrence of gill necrosis are shown in Table 1. The inscription "slight change" indicates that Myxobacteria cannot yet be detected on the gills which might mean that the disease is in its first stage caused by environmental stress, e.g. ammonia, or high temperature.

The next stage, marked "expressed necrotic change" indicates that Myxobacteria are generally but not always present. Only the experimental infections could explain this.

Table 1 Occurrence of "gill necrosis" in fish populations

Date	Age group	Slight changes %	Expressed necrotic change %
1. Oct. 12. 1979	fingerling	35	2
2. Oct. 22. 1979	fingerling	16	5
3. Oct. 23. 1979	fingerling	22	30
4. Oct. 27. 1979	fingerling	-	1
5. Oct. 29. 1979	fingerling	9	6
6. Dec. 8. 1979	fingerling	8	13
7. Sept.29. 1980	fingerling	10	52
8. Oct. 16. 1979	mixed	10	10
9. July 15. 1980	2-year old	9	-
10. Aug. 14. 1980	2-year old	1	3
11. Sept. 9. 1980	2-year old	-	-
12. June 18. 1980	2-year old	5	5
13. Sept.20. 1980	2-year old	3	2
14. Oct. 14. 1980	mixed	2	-
15. Oct. 14. 1980	mixed	-	-
16. Oct. 14. 1980	mixed	4	1
17. Oct. 15. 1980	mixed	5	3
18. Oct. 15. 1980	mixed	1	-
19. Oct. 15. 1980	mixed	5	7
20. April 11.1980	2-year old	anaemia, discolouration	

Myxobacteria could be isolated from 0-10 % of the fingerling population examined with the exception of 2 autumn fishing, when it was much higher.

During the single occasion of the spring evaluation on April 11, 1981 - no Myxobacteria could be detected, but obvious anaemia - discolouration - was seen on the gills. This was experienced at a water temperature of 12-13°C. Environmental stress causing gill damages were experienced in a small pond during a sudden rise of water temperature from 13 to 21°C - sometimes accompanied by Myxobacterial infection. On the full-blooded gills there were small or large dark red spots. This might be regarded a stress initiated stage, which can develop into Myxobacterial

gill disease. These events were very similar to those of experimental heat treatment.

As it can be seen in Table 2, the bacterium flora of healthy and diseased fish gill does not differ significantly. Both Myxobacterium and Streptococcus can be detected at water temperatures higher than 20°C. The difference can only be seen in the number of bacteria which is extremely high on diseased gill. The same is true for Aeromonads.

Table 2 Bacterium flora of healthy and diseased carp gill

Aeromonas hydrophila ssp. hydrophila - biotype 1.
Aeromonas hydrophila ssp. hydrophila - biotype 2.
Aeromonas punctata ssp. caviae
Aeromonas punctata ssp. punctata
Myxobacteria
Streptococci
Acinetobacters
Red Gram negative rods
Yellow Gram negative rods
Other Gram negative rods
Staphylococcus
Actinomyces

The physiological and biochemical properties of Myxobacteria isolated from diseased gill of carp are presented in Table 3. The 8 strains examined are characteristic, long, thin rods with lots of spherical microcysts in older broth culture. The bacteria were Gram negative, gelatin, casein and starch digestion positive. A slight, though not typical cellulose digestion was also experienced. According to the tests performed, the bacterium is Flexibacter columnaris, but this has to be confirmed by serological tests.

Experimental infections were made on 20 occasions. The following disease-process was experienced when smearing bacterium on the gill at a water temperature of at least 20°C. 1-5 hours after smearing the gill lost its colour caused by mechanical injury and could be observed on control fish as well, where no bacterial treatment was applied.

Table 3 Physiological-biochemical properties of Myxobacteria, isolated from "gill necrosis"

Fermentation of glucose, saccharose, lactose	$+_w$
Fermentation of galactose	-
Indol, H_2S, methyl-red test, V-P test	-
Digestion of starch, trybutirin, chitin	-
Degradation of esculin, tween 80	-
Utilization of citrate	-
Digestion of lecithin, casein,	+
Liquefaction of gelatin, catalase	+
Oxidase, digestion of cellulose	$+_w$
Lysis of dead Aeromonas cells	+
Temperature	15-37°C
pH	5.5-9.5
NaCl tolerance	0.1 %

12-24 hrs after smearing, necrosis started and white coating appeared on the smeared part of the gill consisting of large numbers of Myxobacteria. A great proportion of the fish died in this stage of the disease. After 2-4 days the surviving fish started to recover. The coating disappeared in 1-2 weeks and at this stage no Myxobacterium could be detected microscopically, though scarring and distortion remained on the gill. In our opinion this case was analogous to those occurring in natural circumstances where no Myxobacterium could be detected on the distorted gills.

During infection broth cultured bacteria were added in dilution of 1:20 - 1:200 to the bathing water, the infection appeared 4-8 hours later even on the body surface of the fish. We found, however, a correlation between the appearance of the disease and condition of fish and/or virulence of bacterial strain used. Sometimes, even 24 hours of bathing proved to be inefficient.

When the infection was successful, bacteria appeared in high number, the gill discoloured and the animal died. If the bathing was preceded by gill injuring one hour was enough for the symptoms to appear. Fish infected in this way - died.

High mortality caused by Myxobacterial infection could be observed when water temperature was above 20°C and the health condition of the fish was not satisfactory. On this occasion not only the gill suffered serious damage, but ulcers appeared on the body surface. This kind of infection could be prevented by feeding antibiotics - chloramphenicol, oxytetracycline and the one named here "X".

The symptom-complex called "gill necrosis" of carp - caused by Myxobacterium - can be regarded a type of Myxobacterial gill disease. If bacteria are not present on the deformed gill, it can be regarded as recovered stage of Myxobacterial gill disease, but this stage should be separated from gill distortions caused by other diseases.

The symptoms are reproducible with different procedures performed at temperature higher than 20°C. The appearance of the disease mostly depends on the previous and actual condition of the fish as well as on the stress effects, from which the sudden increase of water temperature seems to be the most harmful.

So in the future instead of the terminology "gill necrosis" - we would rather use ammonia intoxication, temperature-, pH-, or other stress caused disease, any of which may cause serious losses and are the preconditions of the Myxobacterial gill disease of carp.

Our opinion is in line with Bullock's, who experienced the same with Myxobacterial disease of trout. During prevention the possibilities of stress effects should be reduced to a minimum and diet containing antibiotics should be applied.

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OBSERVATIONS ON THE CAUSATIVE AGENT OF CARP ERYTHRODERMATITIS IN HUNGARY

GY. CSABA, B. KÖRMENDY, L. BÉKÉSI

Central Veterinary Institute, Budapest, Hungary

ABSTRACT

During the past years more than 30 bacterial isolates of carp erythrodermatitis /CE/ agent were obtained from ulcers of carp originating from various Hungarian fish farms. Successful isolations were primarily carried out from the peripheral area of ulcers on blood agar. Then after 72 h of incubation liquid medium cultures were used for experimental inoculations in the scarified skin of carp using injection needles for this purpose. Infected carp showed characteristic dermatitis 5-6 days after scarification. There were either ulcers, sometimes even in small groups along the line of the scarification, or small sized papulas with hyperemic margins on the sites of needle-pricking, and the central area of the latter was often necrotic. Similar results were obtained after experimental infection of the damaged fins of carp, which then often broke and came off. The pathogenic agent could be easily reisolated from the lesions. The isolates had almost identical features in the bacteriological procedures. According to our examinations these CE agents seemed to belong to Aeromonas genus of Vibrionaceae. More exactly, their bacteriological properties mainly corresponded to those of Aeromonas salmonicida but slightly different from its subspecies.

INTRODUCTION

Infectious dropsy of carp is a disease with an acute and a chronic stage. Results of the last 10 years' research indicate that these two forms should be differentiated on the basis of

etiology. This differentiation was first proposed by Fijan et al. /1972/, who reproduced the transmission experiments of Goncsarov. Fijan et al. /1967/ stated that the infectious material prepared from ulcers, lost its infectivity due to antibiotic treatment. This confirmed that bacteria are responsible for the ulcer formation. Subsequently, Fijan et al. /1971/ isolated a virus /Rhabdovirus carpio/ from the acute cases of infectious dropsy. He named the acute form "spring viraemia of carp" and the chronic ulcerative form "carp erythrodermatitis".

Bootsma /1975/ isolated a bacterium from the ulcerative form, which was able to produce typical ulcers. Later Bootsma and Blommaert /1978/ classified the bacterium as a member of the Aeromonas salmonicida group. It has to be mentioned that Schaperclaus /1979/ referred to the concept of Fijan but he thought the causative agent of dropsy was the Aeromonas punctata. Recently Wiedemann /1980/ isolated a pathogen bacterium from whitefish /Alburnus alburnus L. and Rutilus rutilus L./. This bacterium is related to the causative agent of erythrodermatitis.

MATERIAL AND METHODS

Bacteriological studies on carp erythrodermatitis were carried out on ulcerated carps /Fig. 1/ and in two cases on crucians from fish farms and ponds. Samples were taken from the periphery of the ulcers and from the internal organs. In case of broken fins, a homogenized material was prepared from the hyperaemic parts of the rays and from the little papulas formed on the rays /Fig. 2/. Blood agar plates were inoculated with the above material and incubated at 28°C for 48 h. For further selection of culture media and methods, the requirements of a bacterium strain received from Prof. Fijan at the end of 1975 were taken into account. Biochemical properties of this Fijan isolate and those of the 39 strains isolated by us were investigated as suggested by Cowan /1975/ and Macfaddin /1978/. The shape of the bacteria was studied on Gram stained samples from cultures grown in broth for 48 hours, on blood agar plate for 24 hours, respectively, and incubated on blood agar plate at 10°C for 6 days.

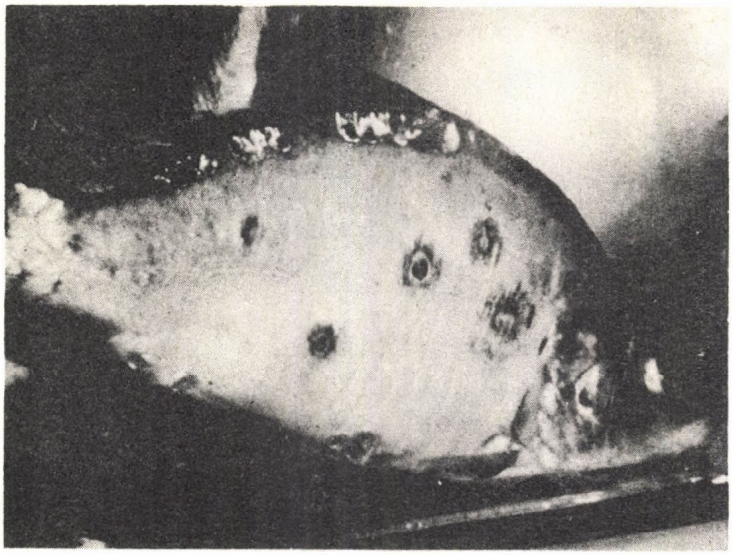


Figure 1 One-year-old carp with typical ulcers of carp erythrodermatitis. The structure of ulcers is well demonstrated with a red central area surrounded by a round zone of white colour and external ring of congestion. At the margin there is a dark area of melanophores

Infection experiments were performed with all isolates as follows: bacterial suspension prepared from 72 hours' agar cultures, or 72 hours' broth cultures were prepared and rubbed into the skin of carps on the site of longitudinal scrapes prepared by a needle. Bacterial suspension was also inoculated into the lesions prepared on the fins as above. The strains isolated from crucians, were also inoculated into the carps. In one experiment grass carps were also infected by removing a few scales and rubbing bacteria into the lesions produced. Fish were infected in groups of 5 and each group was placed into a separate 25 litre areated aquarium. Control and experimental animals were 2 to 3 years old.

Other bacteria, Aeromonas punctata, A. hydrophila and Flexibacter columnaris isolated from the ulcers along with the above strains, were also tested for their infectivity as described above. Pigment producing strains of A. salmonicida isolated from trout, were also used to infect carps. These unlabel-

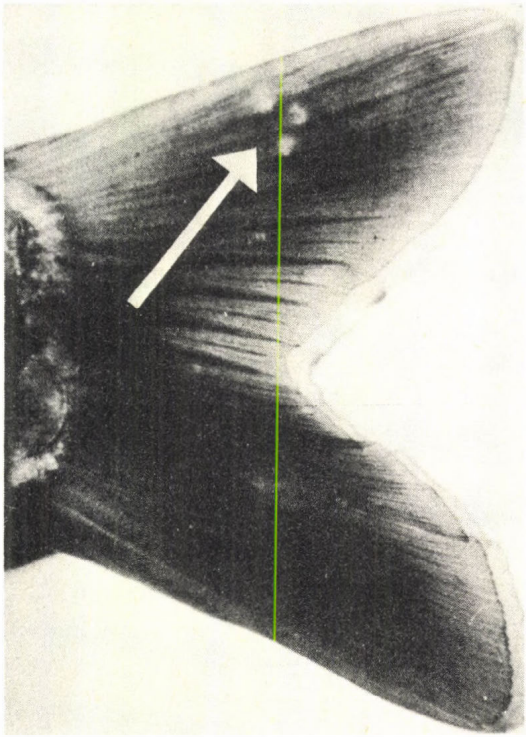


Figure 2 The arrow shows three little papulas on the rays of caudal fin

led A. salmonicida strains originated from Yugoslavia /Prof. Fijan/ and from the German Democratic Republic /kindly provided by J. Farkas, Szarvas, Hungary/.

Therapeutic experiments were performed on experimentally infected diseased carps by adding 50 mg/litre chloramphenicol or the same concentration of Neo-Te-Sol pulvis /containing neomycin and oxytetracyclin/ to their water.

RESULTS

The 37 strains isolated from ulcers of carps and two strains isolated from crucians were all able to cause ulcers on experimentally infected carps. All these bacterial strains were found to have uniform properties: Gram negative, non motile, rod-shaped bacteria with a diameter of 0.4-0.6 µm and with a length of 0.8-1.5 µm. They did not grow on tryptose, McConkey and on Edwards agar. However, they grew well on common agar

/with yeast extract/ with or without serum or blood. There was no bacterial growth at 37°C, but there was some growth at 33°C. At 20 and 28°C small, hardly visible colonies developed on blood agar after 24 hours, and dry beta haemolytic colonies were produced after 48 hours. The same growth was attained in 6 days at 10°C and in 22 days at 5°C. The colonies had a spherical shape and could be removed from the agar surface with a loop in one piece. No pigment production was observed in spite of several weeks of incubation. Colonies were difficult to suspend evenly in physiological saline and bacteria sedimented in a few hours. Staining after White-Wilson /1951/ indicated that all isolates grew in R type of colonies. In broth, granular deposits developed on the side of the tube. These granules were bigger when strains were grown in broth prepared from fish. All isolates - including the one from Fijan - proved to be catalase negative.

All the above strains were resistant to penicillin, oxacillin, methicillin and polymixin B. All were sensitive to chloramphenicol, streptomycin, oleandomycin, tetracyclin, neomycin, erythromycin, oxytetracyclin, nitrofurantoin, sumetrolim, and sulfotrim. Further characteristics of strains are summarised in Table 1.

Based on the above investigations the bacteria can be placed into Vibrionaceae family, Aeromonas genus. Their properties most resemble those of Aeromonas salmonicida but differ from the hitherto described subspecies of A. salmonicida salmonicida, A. salmonicida achromogenes and of A. salmonicida masoucida mainly based on their catalase negativity.

The bacteria were most readily isolated from the peripheral hyperemic parts of the ulcers, but cultures could also be obtained from the papulose lesions of the fins. Cultures prepared from the central parts of the ulcers were negative in most cases: there were only saprophytes. Internal organs were sterile in most cases but in seriously ulcerated fish A. hydrophila and A. punctata were quite often found.

As a result of experimental infection of carps, characteristic dermatitis developed on the 5-6th day after infection and ulcers were formed along the scratching line /Fig. 3/. On skin areas where punctures were made, hyperemic nodules developed

Table 1 Main characteristics of CE agent isolated from carp /37 strains/ and crucian /2 strains/

Gram stain	-	Acid from lactose	-
Morphology	rod	maltose	+
Dimensions: width	0.4-0.6 µm	cellobiose	-
length	0.8-1.5 µm	sucrose	-
Motility	-	sorbitol	-
Oxidase reaction	+	mannitol	-
Catalase "	-	dulcitol	-
Nitrate reduction	+	rhamnose	-
Indol production	-	galactose	-
Urea hydrolysis	-	adonitol	-
H_2S /lead acetate/	+	xylose	-
Voges-Proskauer	+	arabinose	-
Methyl-red	-	glycerol	-
Citrate /Simmons/	-	ONPG	-
O/F test	O/F	Gas from glucose	-
Gelatine liquefaction	+	Amino acids as the only source of carbon	
Hydrolysis of casein	+	L-arginine	-
Starch hydrolysis	+	L-asparagine	-
Acid from glucose	+	L-histidine	-
inulin	-	L-glutamic acid	-
raffinose	-	L-alanine	-
inositol	-		
trehalose	-		

which grew gradually with their central part becoming paler, necrotic and desquamated, resulting in an ulcer surrounded by anhyperemic halo. Bacteria rubbed into the fin lesions resulted in similar nodules /Fig. 4/, finally the fin often broke and came off /Fig. 5/. Fish becoming seriously ulcerated died, showing signs of oedema /Fig. 6/. Bacteria were reisolated from the skin and fin lesions. At the same time no ulcers developed in the controls.

Infection of the two-year-old grass carps, resulted in hyperaemia in the first week on the area where the scales were removed and the inoculation was made. Nodule formation was also observed on the fins. Hyperaemia disappeared, however, quite soon and none of these fish died.

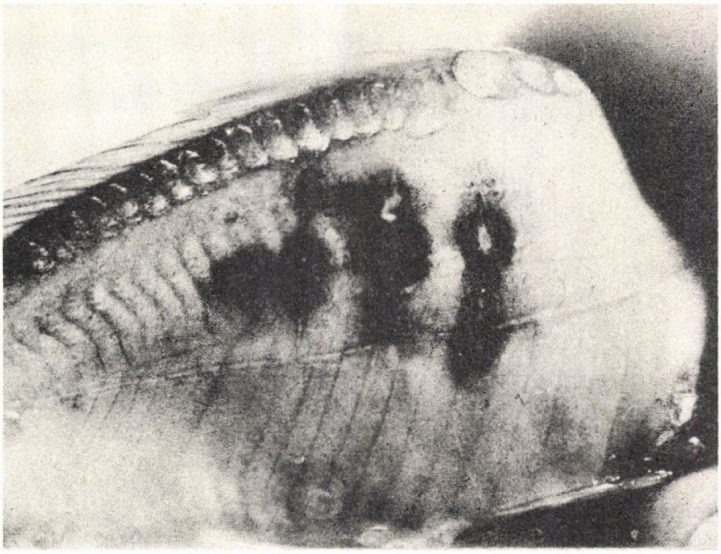


Figure 3 Typical ulcers along the line of scarification is seen on 5-6th days of the experiment

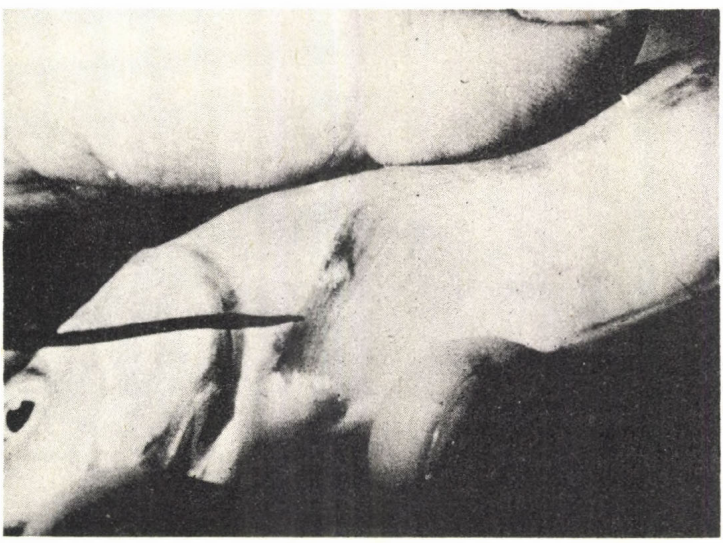


Figure 4 Papula on the fin developed following experimental infection. It is surrounded by congestion and the central part is necrotic

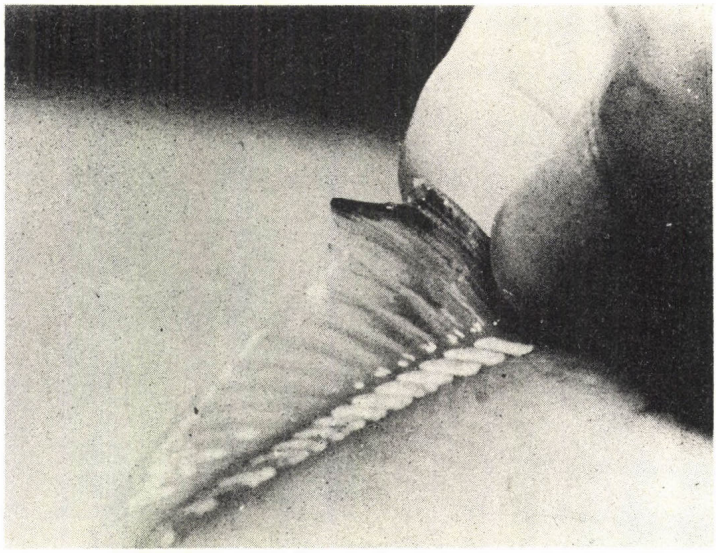


Figure 5 Fin rays often broke and came off at the place of papulas

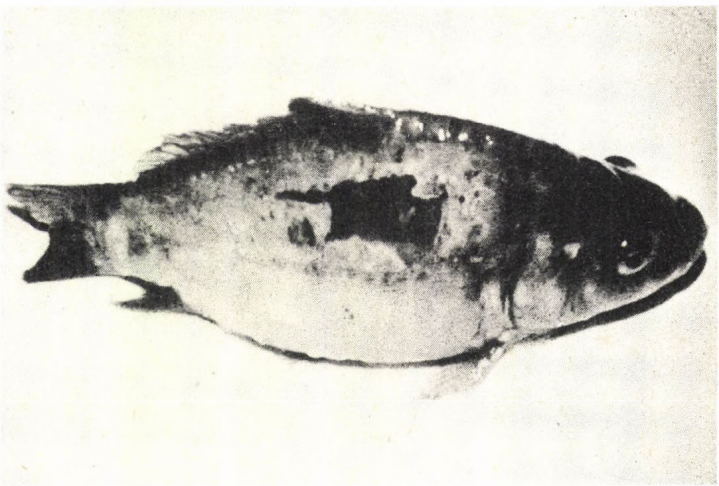
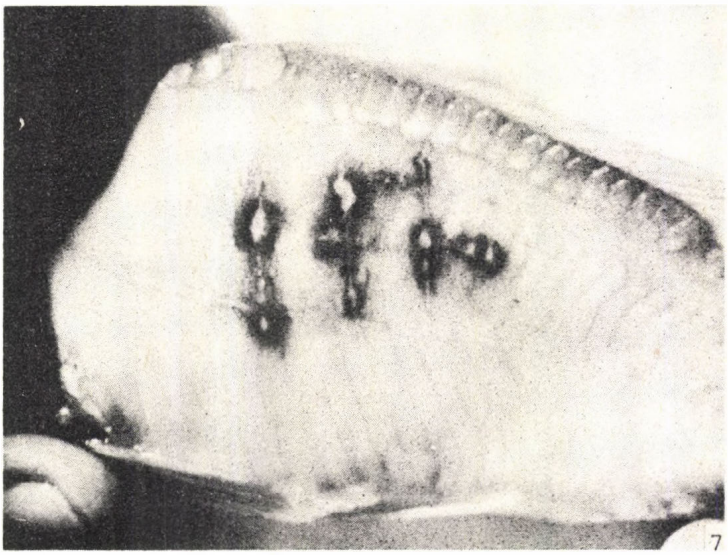
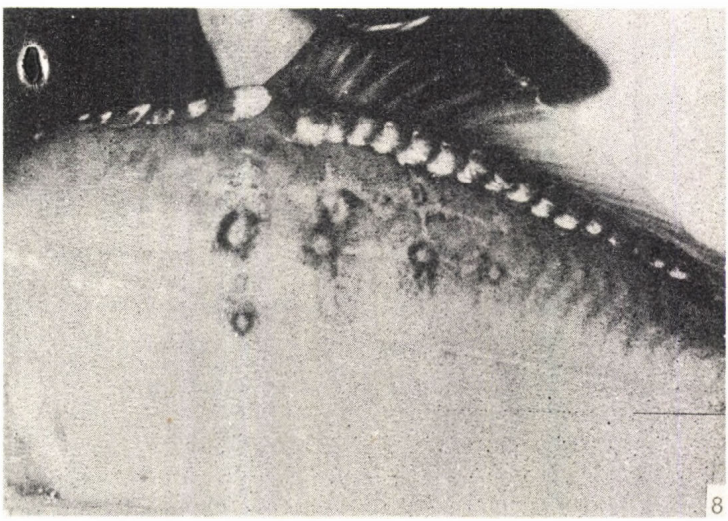


Figure 6 Without treatment serious edema developed with ascites and exophthalmus and the scarified fish died





Figures 7 and 8 The same fish are shown on these figures. The zone of congestion nearly disappeared within 1-2 days following antibiotic treatment

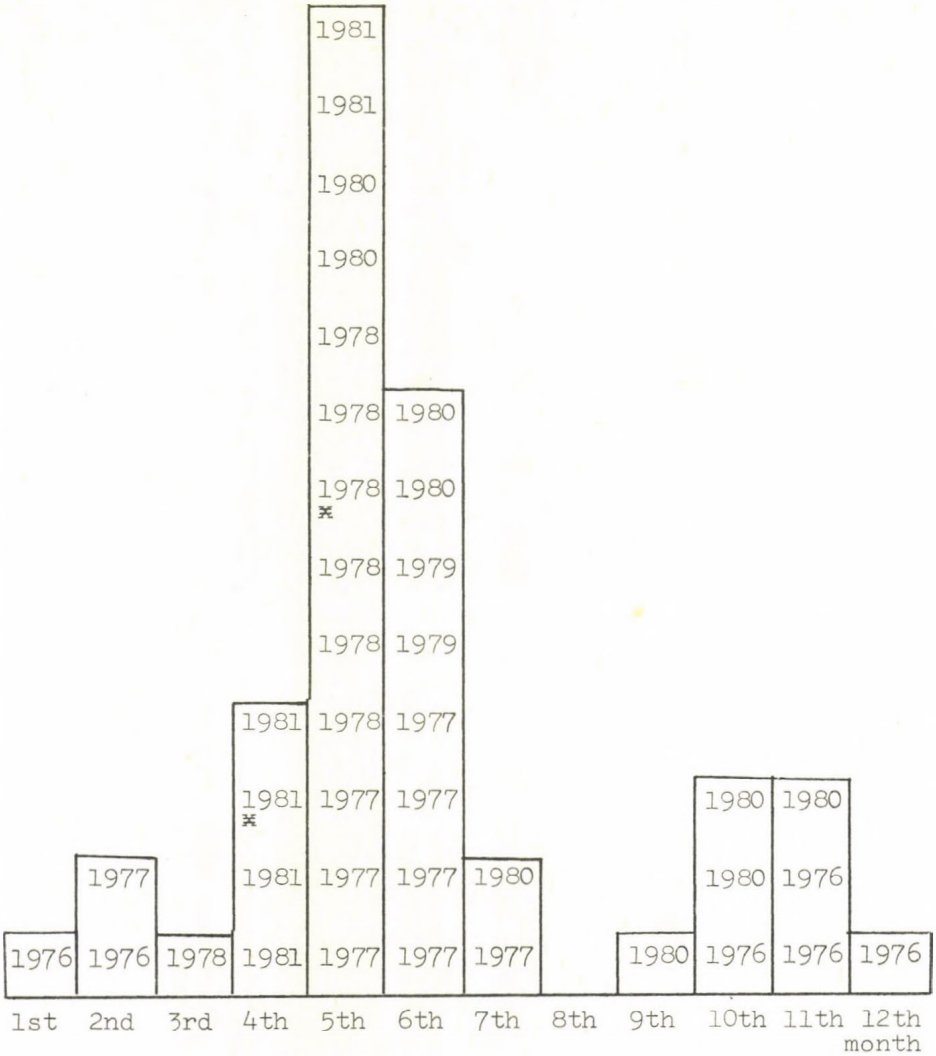


Figure 9 The seasonal fluctuation of the carp erythrodermatitis in Hungary /the isolations of CE agent from 1976 to 1981/ *crucian carp

The strains of Aeromonas hydrophila, A. punctata or Flexibacter columnaris used in these infection experiments did not produce ulcers and neither did the A. salmonicida strains originating from abroad.

Chloramphenicol and Neo-Te-Sol treatments healed the ulcers. The healing procedure was characterised by disappearance of the peripheral hyperaemia followed by epithelialization/Figs 7,8/.

CONCLUSIONS

Our data indicate that all the 37 strains isolated from "carp erythrodermatitis" in Hungary were biochemically and morphologically similar but not identical to Aeromonas salmonicida. The main difference was catalase negativity of the Hungarian isolates. Ulcers, typical of carp erythrodermatitis were produced with all the 37 strains isolated from carps and with two such strains isolated from crucians, when rubbed into the skin lesions of carps, indicating the important role of these bacteria in the pathogenesis of the disease. At the same time, no such lesions could be produced with other bacteria /A. punctata, A. hydrophila, Flexibacter columnaris, and a pigment producer A. salmonicida/ isolated from ulcers.

In Hungary the disease occurs all through the year, however, there is a high prevalence during springtime and fall /Fig. 9/. This observation is supported by Yugoslavian authors, who believe that fish get infected on the selection table at fall when they are sorted out and at the springtime when fishing is also done. These are the occasions when skin lesions can hardly be avoided, and these lesions enable the pathogens to invade.

Isolations of these pathogenic bacteria from the crucian indicate that this fish can play a role in spreading the disease. Results of grass carp inoculation experiments indicate that this species is moderately susceptible to the disease.

Kocylowski and Miaczinski /1963/ believe that the hyperemic edge of ulcers originating from the serous bloody bladders results from tissue overgrowth indicating healing. However, our results contradict this. We feel that the hyperemic edge surrounding the ulcers is due to the presence and activity of the bacteria, because this hyperaemia disappeared after antibiotic

treatment. Our observations indicate that the ulcers are preceded by the formation of nodules, which may lead to the breaking of the fins if they are formed there. Therefore, we believe that it is important to investigate the appearance of the fins continuously at the time of health control studies because broken fins may indicate the presence of ulcer forming bacteria.

It is also interesting to note that no oxytetracyclin resistance was observed among these bacteria in spite of the fact that this antibiotic has been in use in Hungary for several years.

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Symposia Biologica Hungarica 23 (1984)

SEPTICAEMIA IN SILVER CARP (HYPOPHTHALMICHTHYS MOLITRIX VAL.) AND BIGHEAD CARP (ARISTICHTHYS NOBILIS RICH.) CAUSED BY PSEUDOMONAS FLUORESCENS

GY. CSABA, M. PRIGLI**, É. KOVÁCS-GAYER, L. BÉKÉSI, E. BAJMÓCY*, B. FAZEKAS*

Central Veterinary Institute, Budapest,
*Veterinary Institute, Debrecen
**County Veterinary Station, Szeged, Hungary

ABSTRACT

Authors observed a Pseudomonas fluorescens septicaemia in silver carp and bighead carp with high mortality in wintering ponds during 1978-1981. Evidence of bleeding was found on the skin, fins, in the mouth and internal organs of fish accompanied by anaemia. The planktonophagous fishes were kept together with carps in wintering ponds, but the carps did not contract the disease at all. After experimental infection with bacteria isolated from diseased fish, bleeding also appeared all over the body of planktonophagous fishes and they died as a result of infection. The carps had been infected by the same bacteria, but they did not react to the infection. It was found that the bacteria grew well at 4°C, and they were capsulated both in culture and in organs of fishes. Stress factors seemed to play an important role in the pathogenesis of the disease.

INTRODUCTION

The silver carp and bighead carp have been bred for 15 years in Hungary. Both species are very susceptible to injuries which is decreased at lower temperature. Therefore, fishing is done in cold weather before freezing. Injuries due to handling may contribute to secondary pathogenic bacteria entering the organism. With the exception of the work of Bejerano et al. /1979/ there are no data on bacteria causing disease of these fishes. Due to the yearly losses in wintering, we turned our attention to Pseudomonas fluorescens septicaemia causing mortality of silver carp and bighead carp in winter.

MATERIAL AND METHODS

Between January and March during 1978-1981, 30 two- or three-year old bighead carps and 59 silver carps originating from fish farms comprised our material. During these four winter seasons the disease was found in 5 fish farms only among silver and bighead carps and never on carps kept together with them. Haematocrit values were measured and the usual pathological, parasitological and bacteriological examinations were also carried out. Isolation was attempted from the bleeding of epidermis, spleen, liver, kidney and intestine as well as from heart blood. Blood agar and Anacker and Ordal /1959/ media were used for isolation. Smears from spleen, liver, kidney and muscle were prepared at the same time and blood smears were stained according to Giemsa. For histological studies organs were fixed in 10 % formaldehyde, embedded in paraffin and stained with hemalaun-eosin and Giemsa. With the kidney 5 µm frozen sections were stained with Sudan III, too. Identification of bacteria was carried out according to Cowan's /1974/ manual. Novel's /1939/ method was used to detect flagellae. Capsule production of the isolates was demonstrated by Lányi's /1980/ manual. The antibiograms of the isolates were controlled using "Resistest" discs /Human, Budapest/ on standard media according to Lányi /1980/. Isolates incubated at 28°C for 18 hours were used in experimental infection of one summer old, 10 cm long silver carps /15 fish per group/. 0.05 ml of the broth cultures was used for i.p. and i.m. inoculation. Five fish were added to each group as contact controls. Further 15 fish were infected via water. Five carps were also inoculated i.p. with 0.5 ml of broth. Fish were kept in 20 l aquaria that were aerated extensively. Diseased fish were examined as mentioned above.

RESULTS

All the 7 cases investigated, seemed to be identical as far as gross lesions and anamnesis concerned. In most cases the outbreak was preceded by considerable stress some weeks earlier; for example: fishing and transporting at a temperature of below 0°C /in one case at -5°C/. In one case the fish were treated

with a higher concentration of malachite green than usual. On another occasion, an incompetent person opened the lock of the wintering pond and the water current took the fishes out to the field. After collecting them epizoosis appeared due to the evident stress. In three cases stress factors were not detected. Mostly the disease was discovered after thawing of ice on the wintering ponds. Fish were found swimming ashore and near to the surface and they could be caught even with hands. Fish with symptoms died within 12-16 hours. Mortality kept continuing for more weeks causing about 5 % loss a day on average.

It was possible only once, to find severe Dactylogyrus invasion otherwise, the number of Ciliata found were limited. On the epidermis of both species hemorrhages of different size could be seen. Bleedings were found all over the body and around the base of fins, especially on the pectoral ones. In some instances the anus was protruded with the reddening of perianal tissue. In such fish a discharge containing blood was detected when pressing the abdomen. Petechia on the head, around the mouth, in the eyes, in the oral cavity were frequently seen /Fig. 1/. Hemorrhages on the inner surface of opercula and on the mucosa of palatinum were the most characteristic signs /Fig. 2/. Bleedings occurred one by one or in groups. Gills were always pale and anemic. In the third part of the intestine, exudate was often found and petechia were seen even in the serosal site. Hemorrhages were discovered in the liver, heart and in the wall of swim-bladder. In some instances small focal necrosis was seen in the liver. Blood was very pale and the hematocrite values varied below 10 %.

In Giemsa stained smears prepared from different organs and blood, capsulated bacteria were seen in great number. Bacteria were surrounded by an eosinophilic capsule /Fig. 3/. Histopathology revealed hemorrhages in every organ. Focal necrosis and severe edema were seen in the liver with the diffusion of hepatocyte lines /Fig. 4/. Brown pigments also appeared occasionally. Degeneration and disintegration of tubuli in focal area were found in the kidney with cytoplasm degeneration of cells containing eosinophilic debris that were not stained by Sudan staining /Fig. 5/.

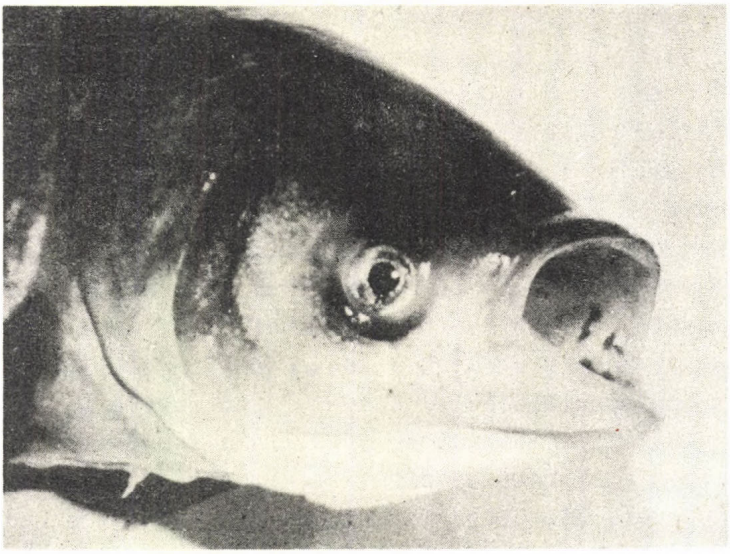

Figure 1 Hemorrhages in the eye and mouth of a silver carp

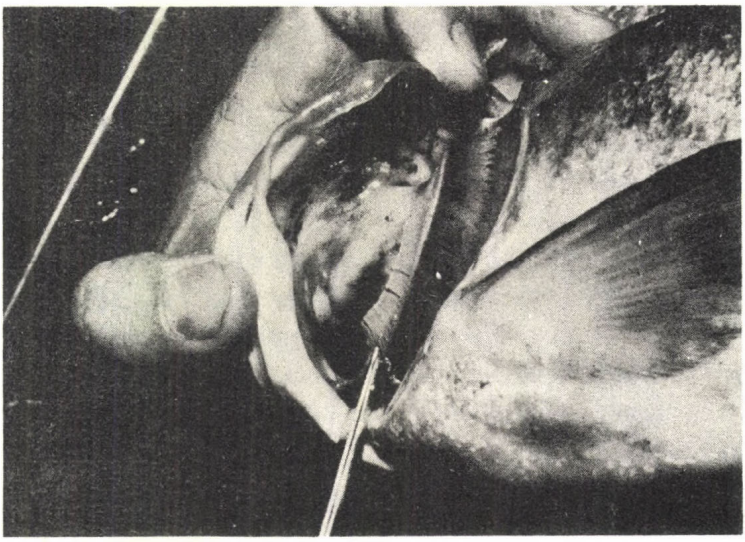

Figure 2 Bighead carp with petechia on the mucosa of operculum and palatinal folds

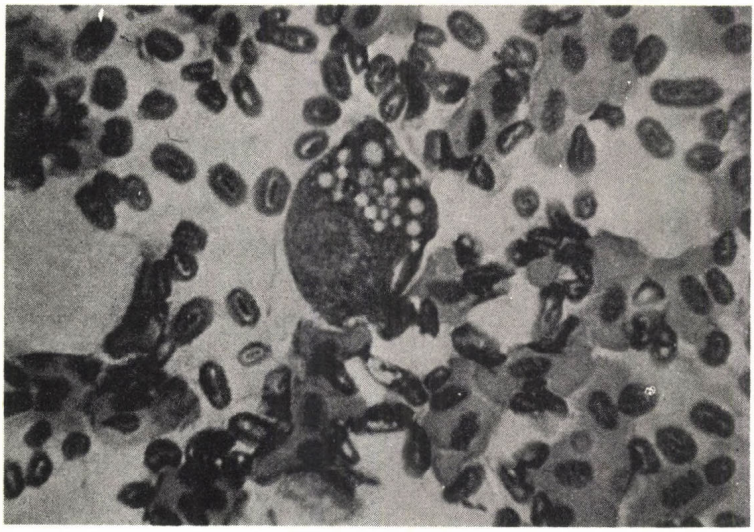

Figure 3 Many bacteria with eosinophilic capsules from kianey smear /Giemsa staining, 1,500 x/

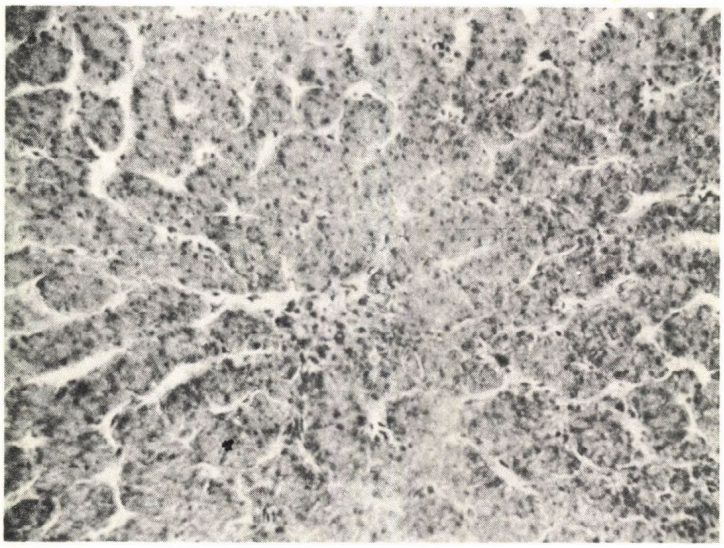

Figure 4 Severe edema in the liver with diffusicn of hepatocyte lines /Hemalaun-eosin, x 250/

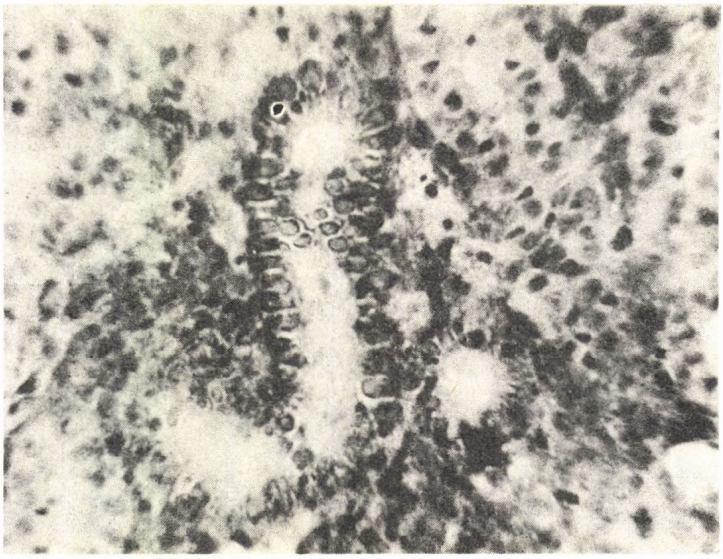

Figure 5 Tubulonephrosis in the kidney with eosinophilic debris in the lumen /Hemalaun-eosin, x 400/

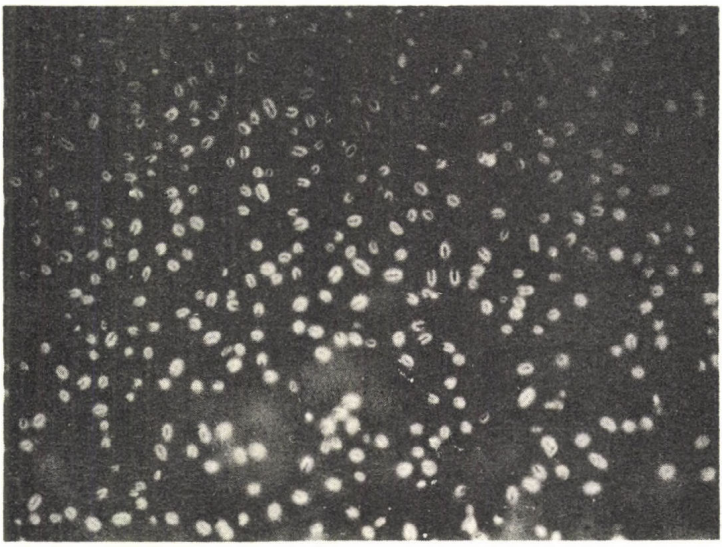

Figure 6 Capsulated bacteria from the culture /Ink technique, x 600/

Table 1 Main characteristics of *Pseudomonas fluorescens* isolated from silver carp and bighead carp /based on 7 strains/

Test at 28°C	
Gram stain	-
Morphology	rod
Dimensions	0.8 x 2.9 µm
Growth	+
at 4°C	+
at 37°C	-
Fluorescent pigment	+
Other pigments	-
Motility	+
No. of flagella	1-4
Oxidase	+
Catalase	+

OF	Oxidative/not	Fermentative
Citrate: Simmons	+	
Gelatinase	+	
Urease	-	
Indole	-	
H_2S	-	
MR	-	
VP	-	
ONPG	-	
Starch hydrol.	-	
2,3-butanediol dehydrogenase	-	
Acid from glucose	+	
trehalose	+	
inositol	+	
sucrose	+	
sorbitol	+	
adonitol	-	
L-arabinose	+	
maltose	+	/2 strains:-/
mannitol	+	
salicin	-	
xylose	+	

From every organ and blood sample Pseudomonas fluorescens bacteria were isolated in homogenous cultures. These were found in mixed flora in gut, but in some cases they grew in homogenous culture. Isolated strains of Pseudomonas fluorescens could be regarded as identical. Our strains formed yellow-brownish, smooth, mucoid colonies in 24 hours on the media applied. Limy thread could be drawn by a loop from the colonies. We could not find such capsulated forms in Giemsa stained smears from colonies seen in smear of the organs and blood. Capsule production was determinated by negative staining /Fig. 6/. Further characteristics of bacterium are summarised in Table 1. Antibiograms are shown in Table 2.

Table 2 Antibiogram of Pseudomonas fluorescens strains isolated

Oxacillin	R	Erythromycin	R
Methicillin	R	Nitrofurantoin	R
Penicillin	R	Chlortetracyclin	R
Chloramphenicol	R	Oxytetracyclin	R
Oleandomycin	R	Vancomycin	R
Streptomycin	S: 3 strains MS: 4 strains	Kanamycin	S
Tetracyclin	MS	Spiramycin	R
Neomycin	S	Novobiocin	R
Polymyxin B	MS		

S: sensitive; R: resistant; MS: moderately resistant.

All the fish inoculated i.p. or i.m. in our experiment died in a period of 10 days. The day before death, fishes moved slowly, their body became dark and hemorrhages appeared on the fins and in the oral cavity. Hemorrhages were also found in the organs by post mortem examination and the histopathological changes were similar to those in natural cases. In the smears of organs the same capsulated bacteria were demonstrated by Giemsa-staining and the same ones were reisolated. Water-borne infection could not be induced by broth culture and the contact controls also remained healthy during the one month experiment. Carps inoculated i.p. survived the infection without symptoms.

DISCUSSION

No capsulated Pseudomonas fluorescens bacteria have been isolated from silver carp and bighead carp thus far. From other fish species Bullock /1965/ described a Pseudomonas fluorescens causing hemorrhages in goldfish which were characterised also by capsule production and lack of motility. The motility of our isolates was demonstrated by means of dark field microscopy since the use of semisolid medium did not give any definitive result. According to our experience, lesions such as erosions and hemorrhages caused by fishing, and transportation must be differentiated by low mortality and lack of petechia in the oral cavity. In cases, where bleedings were found in the oral cavity, Pseudomonas fluorescens bacteria could always be isolated.

In our experimental infections the high susceptibility of these fishes to these bacteria were demonstrated in comparison with carp which we could not infect either naturally or experimentally. Experiments carried out made it clear that the possibility of penetration of the bacteria is a precondition of the infection. All these facts suggest that erosions, injuries during handling and different stressors play an important role in the pathogenesis of the disease. The psychrophilic character of the Pseudomonas fluorescens bacteria /growing at $4^{\circ}C$ well/ is also a promoting factor of this disease. Due to the alimentary character of these fishes the use of medicated feed is hopeless in spite of having antibiograms. For prevention of the disease careful handling and elimination of stressors would have to be considered.

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PARASITES OF FISHES

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SOME PECULIARITIES OF OOCYST REJECTION OF FISH COCCIDIA

K. MOLNÁR

Veterinary Medical Research Institute,
Hungarian Academy of Sciences
Budapest, Hungary

ABSTRACT

Most coccidia leave the fish in sporulated stage. Besides the few exceptions which are shed non-sporulated, there are species which exhibit the possibility of rejecting viable oocysts in non-sporulated and sporulated stage as well. Whether sporulated or non-sporulated oocysts are shed, is mostly regulated by the species itself, but it is also influenced by the location in the fish and by the host reaction. Species developing close to the intestinal lumen can easily leave the fish non-sporulated, but others inhabiting deeper layers or inner organs always finish sporulation inside the host. In case of Eimeria species developing in epithelial cells of the intestine, as a result of regeneration, the oocyst containing invaded cells overgrown by the uninfected ones are pushed into the deeper layers. In this case the rejection of oocysts is accomplished only secondarily. In inner organs where the rejection is impossible, the oocysts leave the fish only after the death of the host, unless they are destroyed by RES macrophages.

INTRODUCTION

Eimeria species of fish differs in many respects from those living in mammals and birds. Some of these are thoroughly dealt with by Schulman /1962/, Lom /1970/ and Molnár /1977/. The most obvious characteristics of fish coccidia seem to be the following: very thin wall of oocyst, long developmental cycle depending on the temperature, majority of species leaves the fish in sporulated stage.

The thin wall of oocyst in fish coccidia can be attributed to their environment, i.e. that in the water there is no need for a resistant envelope. The relatively long developmental period of fish coccidia can also be partially explained by the fluctuating temperature of the fish. At low temperatures the duration of development is longer while at high temperatures it becomes shorter. Zmerzlaya /1966/ who examined E. carpelli, found that the oocysts were first discharged on the 17th day after infection at 17°C, and on the 7th day at 20°C.

The majority of the mucosa dwelling species in the fish gut with an oocyst diameter of 8-14 µm /E. carpelli, E. sinensis, E. cyprinorum, E. iroguoina etc./ follows a continuously repeating developmental pattern, which is influenced by the temperature, but does no actually depend on the season of the year. Other fish coccidia, first of all species with an oocyst diameter of 17-30 µm /E. subepithelialis, E. metschnikovi, E. cylindriformis, E. degiustii etc./, develop by an annual cycle and only one generation is formed in a year. Marincek /1973 a/, who experimentally studied the developmental cycle of E. subepithelialis explained this phenomenon as a hibernating effect which would be necessary to provoke gamogony.

Most coccidia leave the fish in a sporulated stage. This is a well known fact /Schäperclaus 1954, Schulman 1962, Kheysin 1972 etc./ the cause of which, however, has not been studied so far. There are only a few exceptions like E.pigra described by Léger and Bory /1931/, and E. aurati described by Hoffman /1965/, which are shed in a non-sporulated stage or E. micropteri described by Molnár and Hanek /1974/, which leaves the fish semi-sporulated. Marincek /loc.cit./ took the first step in this respect, when she proved that E. subepithelialis can be shed either in a sporulated or a non-sporulated stage, depending on the pathophysiological situation of the fish gut.

On the basis of observations made on different Eimeria parasites of fish, this paper presents data to show that the location of the parasites in tissues, the duration of development, the intensity of infection in the host and the tissue reactions affect rejection of either sporulated or non-sporulated oocysts.

In the development of Eimeria species of warmblooded animals there is an endogenous period above 37°C less suitable for sporu-

lation and an exogenous one when the temperature is significantly lower. Similar temperature factors affect Eimeria oocysts inside and outside the fish body in case of cold blooded fishes. This means that whether sporulation takes place inside or outside the fish, mostly depends on the speed of oocyst rejection. It is obvious that species like E. pigra, which develops on the surface of the intestinal epithelium can easily be detached from epithelial cells, and therefore, the oocysts leave the intestinal canal fast and unsporulated. On the other hand, it seems reasonable that oocysts of species like E. metschnikovi and E. degiustii living in the spleen of Cyprinids or E. gadi inhabiting the swimbladder of Gadidae can escape from fish organs only after the death of the host, and therefore, they sporulate during the long period spent in the host. Similarly, oocysts of E. sardinae in the testes, or E. leucisci and E. rutili in the renal tubuli have plenty of time for sporulation until they are shed to the outer world with sperm or excretion. Some species, like E. subepithelialis, E. anguillae exhibit the possibility of rejecting oocysts in non-sporulated and sporulated stage as well. These parasites serve as excellent models for studying the problem of oocyst rejection.

The results of my experiments which agree with those of Marincek /1973 a and b/, show that the gamogonic development of E. subepithelialis, causing the nodule coccidiosis of common carp starts in early spring /March or April/ in the intestinal epithelium involving some neighbouring intestinal folds. In these sharply demarcated parts of the gut almost every epithelial cell is invaded by micro- and macrogametocytes /Fig. 1/. In the centre of the nodule the infection involves the tip and the bases of the folds as well. Towards the edge of the nodule, however, the infection is mostly restricted to the tip of the folds. The future of the young oocysts developing in epithelial cells can proceed in two different ways. The mechanically most affected oocysts inhabiting the cells on the tip of the folds, are freed from the desquamated epithelial cells by peristalsis, and imbedded in the intestinal mucus they are shed non-sporulated in high numbers in the faeces as early as in April. The fate of oocysts remaining in the intestinal cells is different.

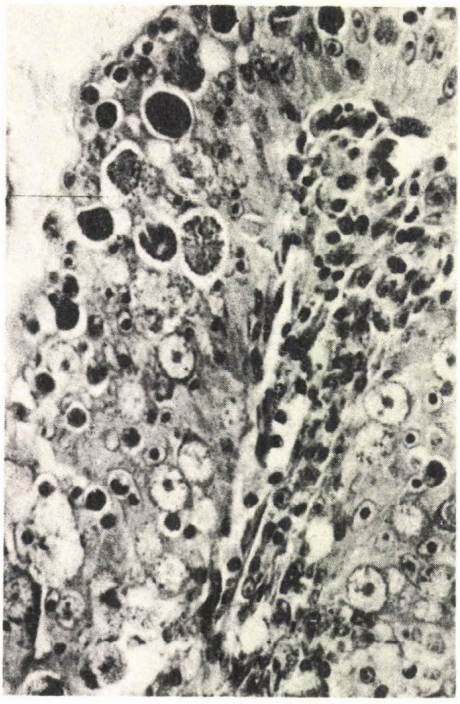

Figure 1 Nodule coccidiosis in common carp. Micro- and macrogametocytes in the epithelium. H. and E. x 1,200

Figure 2 Nodule coccidiosis in common carp. Sporulated oocysts in the submucosa covered by regenerated epithelium. H. and E. x 200

In Marincek's /1973 b/ opinion these oocysts sink into the deeper layers and they form nodules in the submucosa /Fig. 2/. In my opinion the mechanism of sinking into the deeper layers is a consequence of active defensive reaction of the host. Epithelial cells attacked by young developmental stages can perform their duties for a while, although they do it in a decreased manner. After the formation of oocysts the degenerated cell cannot provide food requirements anymore, and a host reaction starts to replace the defective cells. The regeneration of the epithelium begins with proliferation of the intact epithelium surrounding the nodules, but some uninfected epithelial cells randomly occurring among the infected ones, can also serve as a bud of regeneration. The uninfected epithelial cells overgrow the oocyst containing regenerated ones and form an intact contiguous layer over them. The oocyst mass embodied by disintegrating epithelial cells is pushed step by step into the submucosa, where the oocysts released from the cells will be surrounded by a loose connective tissue of the submucosa and demarcated just above the muscular layer. In the course of demarcation which takes about one month, the oocysts finish sporulation. The oocyst mass containing less and less tissue elements behaves like a foreign body, the monolayered epithelium above it secondarily dies or becomes damaged, and finally the mass of sporulated oocysts is rejected into the gut. In this way about the end of May some more oocysts of the same Eimeria species are shed, but this time in sporulated stage.

In the course of diffuse enteric coccidiosis caused by E. carpelli in common carp, or E. sinensis in silver carp a similar, though partly different mechanism appears in the host-parasite relation. The infection with these non-seasonal, continuously developing species is characterised by a symptom, where at the same time, different developmental stages occur in the epithelium and they develop in cells located relatively distant from each other. Around the invaded cells, intact cells can also be found, from which the local regeneration starts. These cells quickly surround the oocyst containing cells which are unable to function, overgrow them and push them into the propria or submucosa layers /Fig. 3/.

Figure 3 Diffuse coccidiosis in common carp, caused by E. carpelli. Sporulated oocysts are located mainly in the propria layer of the gut. H. and E. x 500

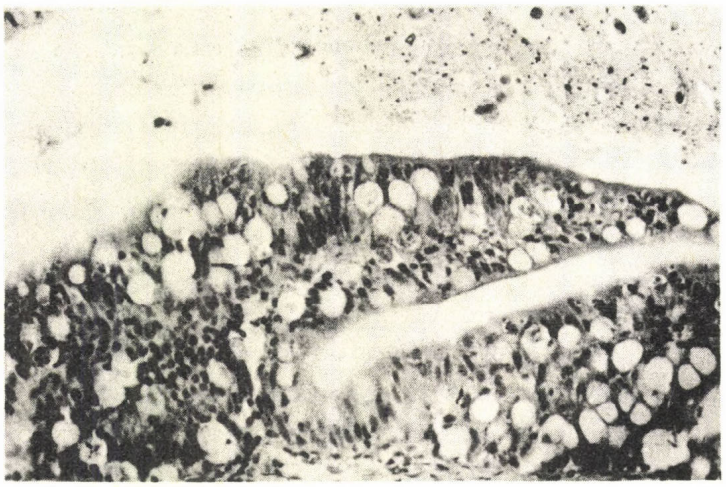

Figure 4 Diffuse coccidiosis in silver carp, caused by E. sinensis. Sporulated oocysts are mainly located in the propria and submucosa layers. Young trophozoites are in the epithelial cells. H. and E. x 500

It has been proved by histological examination that sporulated oocysts can practically be found only in the propria and submucosa in these species as well. At high infection levels, development of new trophozoite generations can be observed in the regenerated epithelium above the oocysts in the submucosa /Fig. 4/. The demarcation of oocysts can be observed both in E. carpelli and in E. sinensis, but this process is restricted only to some /3-7/ oocysts. An essential difference between the two species seems to be that the oocysts of E. sinensis get completely free from the disintegrated epithelial cells, while the demarcated oocysts of E. carpelli are encased in so called "yellow bodies" comprising 3-7 oocysts and contain a yellow pigment material, presumably iron. The yellow body originates probably from serum and debris of decomposed cells. The ageing of oocysts in the submucosa continues even after sporulation. In native samples the ageing of the oocysts is marked by the continuously decreasing sporocyst residuum, while in histological preparations stained by Farkas-Mallory's method the sign of ageing is an intensive yellow colour of the oocysts. /The young oocysts stain blue or red with this method./ In the same way, as in the case of nodule coccidiosis it is supposed that only mature oocysts take up a foreign body character. The regenerated epithelium above them dies secondarily and the oocyst will be rejected from the tissues by the mechanical effect of the peristalsis. This course proceeds, however, in small locations and with great variations in time. With intensive infection the demarcation of oocysts is less characteristic, and from the intensively damaged epithelium relatively young oocysts are released. In this case free oocysts unembedded in yellow bodies can often be observed even in E. carpelli invasion.

A significantly different mechanism of host reaction occurs in Eimeria species inhabiting the inner organs, like E. degiustii, E. metschnikovi in the spleen, E. scardinii in the renal parenchyma or E. siliculiformis in the mesentery. These parasites can live in the host for a long time without provoking any host reaction. Coccidia of this type are supposed to develop by a one year seasonal cycle. The young oocysts start their development in the spring and after sporulation oocysts consuming their abundant sporocyst residuum can survive several months. In the case

of E. metschnikovi, the concentration of the dispersely developing oocysts into islets containing 100 to 600 oocysts can be regarded as the first sign of host reaction. The concentration and demarcation of oocysts are carried out by the epitheloid type RES marcophages. These cell types are commonly found in the parenchyma of the healthy spleen as well, but they are always abundant around the moribund oocysts. These RES macrophages in the spleen, which contain yellow pigment, dissolve and destroy the old oocysts step by step /Fig. 5/. In species developing in the mesentery /E. siliculiformis, E. cobitidis/ the appearance of RES macrophages is also common, they, however, contain usually black pigment.

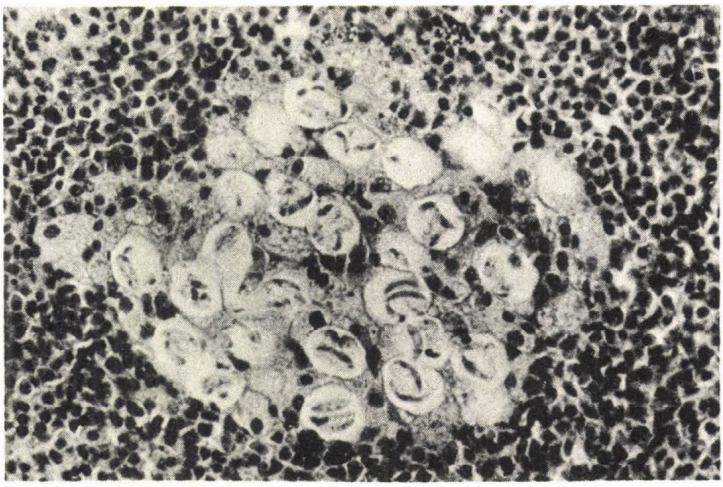

Figure 5 E. metschnikovi infection in the spleen of gudgeon. The oocysts are surrounded by melanomacrophages. H. and E. x 1,000

Both in E. metschnikovi in the spleen, and E. scardinii in the kidney parenchyma a demarcation of oocysts by connective tissue can also be observed. This fibrotic capsule is usually formed around oocyst masses containing older oocysts /Fig. 6/, but sometimes it surrounds young oocysts as well /Fig. 7/. Sclangi and Overstreet /1980/, who studied E. funduli infection in the liver of killifishes also observed fibrotic capsules around clumps of oocysts and concurrently the appearance of yellow or dark pigment.

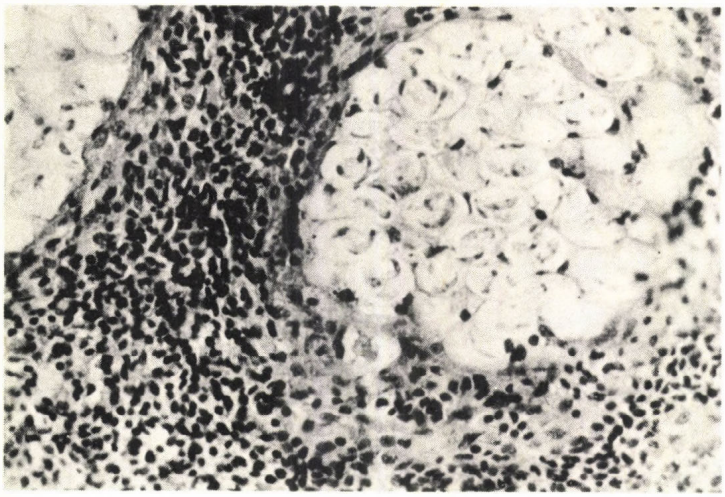

Figure 6 E. metschnikovi infection in the spleen of gudgeon. The sporulated oocysts are surrounded by a fibrotic capsule. H. and E. x 1,000

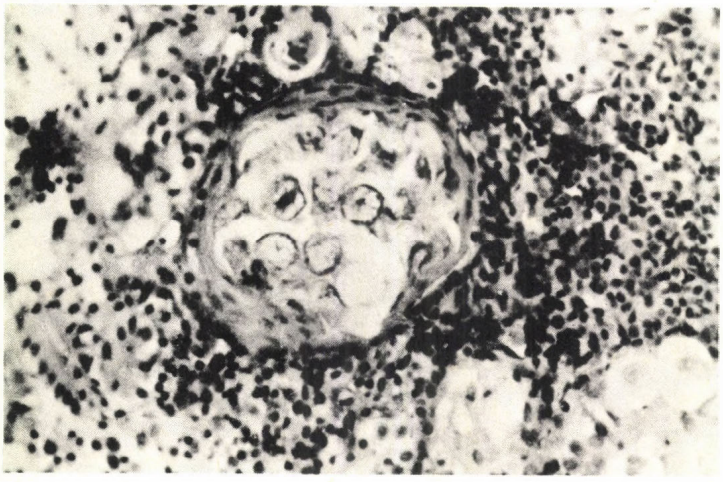

Figure 7 E. scardinii infection in the kidney parenchyma of Chondrostoma nasus. Young, non-sporulated oocysts are surrounded by a fibrotic capsule. H. and E. x 1,000

As no iron was histochemically detectable in the yellow pigment they regarded this material as lipofuchsin. Similarly, they could not identify the black pigment as melanin. Solangi and Overstreet /1980/ described an infiltration by inflammatory cells around oocysts as well. In our case we did not see infiltration and neither did Odense and Logan /1976/ during their study on a heavy swimbladder coccidiosis caused by E. gadi.

No experimental data are available on the transmission of coccidia inhabiting the inner organs of fish. It seems, however, to be likely, that after the death of the host, even if the fish was consumed by predators, oocysts preserve their viability in the outer environment, unless the sporocyst residuum has essentially decreased. The possibility that oocysts of fish coccidia can survive passage through the alimentary canal of carnivores is supported by the observations, the Eimeria species from sea fishes such as E. sardinae and E. clupearum pass through the alimentary canal of man morphologically well preserved /Schulman 1962/.

Fish coccidia studied showed a very high level of adaptation to hosts and as long as their intracellular development has not finished they are not immunogenic. The defensive reaction of the host starts with the ageing of oocysts and with the disintegration of the infected host cells, and depends on the host species and location of the parasite while host reaction is manifested either in encapsulation or in rejection of the oocysts.

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PATHOGENICITY OF SOME PROTOZOAN PARASITES OF CYPRINID FISHES

J. LOM, I. DYKOVÁ

Institute of Parasitology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

ABSTRACT

Pathogenic protozoa of carp fingerlings in South Bohemian region of Czechoslovakia are reviewed. Special attention is paid to myxozoan parasites. Sphaerospora sp. develops in the stratified epithelium of gill filaments but also in the double layer of epithelial cells covering secondary lamellae causing serious, respiration impairing lesions. Chloromyxum cyprini Fujita, 1927, known to inhabit gall bladder, may invade the liver tissue in carps and grass carps causing massive necrosis. Four myxozoan species were found to infect carp kidneys. Two of them were recorded in Czechoslovakia for the first time, i.e., Sphaerospora sp./a species different from that found in gills/ and Mitraspora cyprini Fujita, 1912. They were detected in 65 % and 21 % of the carp stocks examined, respectively. Both species live in the lumen of the renal tubuli, but have early intracellular stages within the tubular epithelium and provoke distinct histopathological changes of the tubuli. Structures tentatively classified as developmental stages of Hoferellus cyprini Doflein, 1898 were found in 17 % of the surveyed ponds. They develop intracellularly in the epithelium of convoluted tubuli. Large accumulations of the parasites finally rupturing the membrane of tubuli are released into the interstitium resulting in a granulomatous inflammatory reaction. Myxobolus cyprini Doflein, 1898 was found on 34 % of surveyed localities. No pathogenicity was recorded.

INTRODUCTION

Of the many protozoan species known to infect carp /totalling now 84 species - see also Lom et al. 1976, Margaritov 1976, Reichenbach-Klinke 1980/ the present day carp fishery is concerned with those which may become important pathogens in water reservoirs and ponds densely stocked with carps. In intensive carp cultures with high density of fish fed artificial diet, requiring often aeration or other technological interventions, environmental conditions may easily prevent the resistance of the fish organism. Also, the spread and course of various protozoans can be quite different from those in natural waters or in low intensity fisheries.

It is worthwhile drawing attention to some recent developments in our knowledge of some groups of endoparasitic protozoa potentially pathogenic to the culture of carps and some other cyprinid fishes in Central Europe. Protozoans invading the body surface of fish such as Ichtyobodo, Chilodonella or Ichthyophthirius - although incontestable pathogens - are outside the scope of this paper.

MATERIAL AND METHODS

Material for our studies came from the region of Southern Bohemia, Czechoslovakia, a home of highly developed carp culture for centuries. During 1979 to 1981, about 750 carp fingerlings and 50 second year carps were examined for the presence of protozoan parasites. To detect protozoa invading the surface, fresh scrapings from the skin and gills were examined. To reveal the endoparasitic protozoa, macroscopical inspection was followed by examination of fresh tissues from body organs and of fresh contents of their cavities. Tissue samples were also fixed and histological preparations examined. Our experiences proved a satisfactory correlation between findings of histozoic protozoans in squash preparations of tissues and on stained tissue sections; this applies especially to myxozoa inhabiting renal tubuli.

RESULTS

Blood flagellates

Trypanosoma danilewskyi Laveran and Mesnil, 1904 and Trypanoplasma borelli Laveran and Mesnil, 1902 /syn. T. cyprini Plehn, 1903/ are common parasites in carp stocks. Over the last three years the infection has declined due to improved control of leeches in the area. The carp trypanosome, T. danilewskyi, has been hitherto considered harmless. Preliminary results with experimentally infected carps indicate that this species may be pathogenic for carps, too /Lom 1979/. Its high pathogenicity for experimentally infected goldfish has been established by Dyková and Lom /1979/. Experiments are in progress to elucidate pathogenicity of T. danilewskyi for young carps.

Trypanoplasms have long been suspected of causing diseased conditions in fish, which was finally proved for T. salmositica /Putz 1972, Woo 1979/ and for T. borelli /Lom 1973/. The pathological changes in goldfish experimentally infected with T. borelli were described by Dyková and Lom /1979/. Preliminary findings on the pathogenicity of T. borelli in experimental infections of carp fry and fingerling have to be verified. In South Bohemian region trypanoplasma infections have been repeatedly found to be associated with diseased condition of fingerling and second year carps. In some cases, lesions in the kidneys may reach macroscopical size.

Coccidia

Eimeria carpelli /Léger and Stankovitch, 1921/ and Goussia /syn. Eimeria/ subepithelialis /Moroff and Fiebiger, 1905/ are wide-spread in Czech carp stocks; about 87 % and 14 % of the carp stocks examined were infected with E. carpelli and G. subepithelialis, respectively. Although it seems that no direct losses were incurred due to carp coccidioses, the deleterious impact of infection on the fish growth cannot be underestimated.

Data on coccidia of other commercially important fish are rather scarce and there may be new important facts to be discovered. Dyková et al. /1981/ have observed the development of an Eimeria sp. in kidney and gill secondary lamellae of roach /Rutilus rutilus/. This species differs from E. rutili as orig-

Figure 1 A zygote of Eimeria sp. within the gill secondary lamella of roach /Rutilus rutilus/. Note the large central chromophil inclusion. H.-E., x 1,400

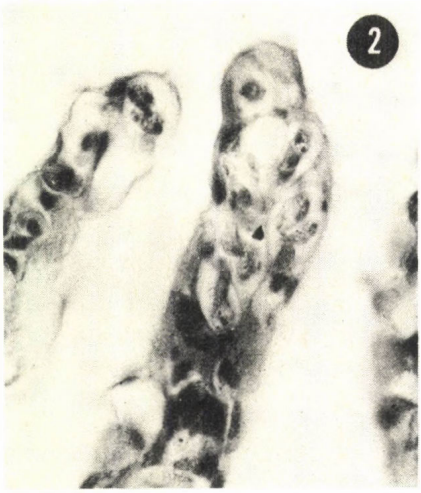

Figure 2 Mature sporocysts of Eimeria sp. in the blood spaces of the gill secondary lamellae. H.-E., x 900

inally described by Dogiel and Bykhovsky /1939/ and from other coccidia recorded from roaches and related cyprinid hosts by Molnár /1978/, Molnár and Pellérdy /1970/ and Pellérdy and Molnár /1968/. The site of schizogony and gamogony is not known, while sporogony starting from what appears to be a zygote /Fig. 1/ takes place in kidney and gills. Mature oocysts were found in the blood spaces between pillar cells. Biological significance of this strange site of sporogony can be seen in an easier spread of sporulated sporocysts into the environment when the capillaries clogged by sporocysts /Fig. 2/ rupture and release the sporocysts to the outside.

Myxozoa

Of the many species infecting carps there are several that deserve special attention because of the newly disclosed facts on their spreading, pathogenicity and patterns of life cycle, such as causative agents of gill sphaerosporosis of carps, hepatic chloromyxosis of carps and grass carps, and renal myxozooses of carps.

1. Gill sphaerosporosis of carps

This infection was found in about 5 to 7 % of the carp stocks surveyed. The Sphaerospora species found could not be easily identified with Sphaerospora carassii Kudo, 1919 in contrast to Hungarian authors who reported it from grass carps /Molnár 1971/, carps /Buza, Hámory and Sziklai 1971; Hámory and Molnár 1972/ and from both fish hosts /Molnár 1971/. Pavlásková /unpublished observation/ has found Sphaerospora sp. frequently located both in gills and in scrapings of epidermis on the head and in the nasal pits.

In the gills, developmental stages and mature spores of Sphaerospora sp. were located usually in the stratified epithelium causing its dystrophic changes and necrosis. In massive infections, the parasite almost completely replaces the interlamellar cell layers. Necrosis of the epithelial cells surrounding numerous aggregations of maturing spores makes the release of spores from the infected tissue possible.

Contrary to the report of Molnár /1979/, we have found aggregations of mature spores not only in the stratified epithelium of the gill filaments but also in the double layer of epithelial cell covering secondary lamellae, usually between the

Figure 3 Mature spores of Sphaerospora sp. in the gill secondary lamellae of carp. H.-E., x 300

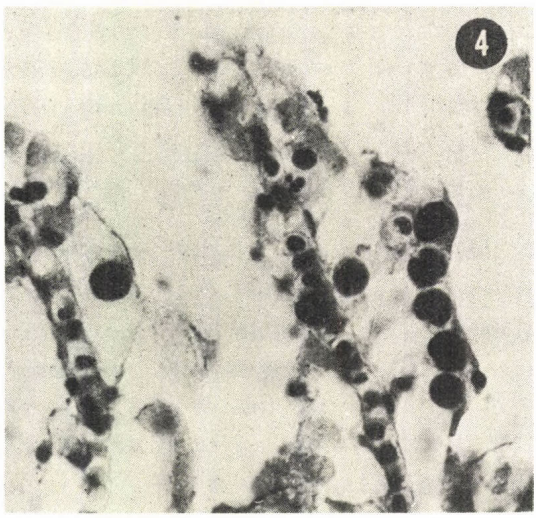

Figure 4 Two secondary lamellae harbouring spores of Sphaerospora sp. under their epithelium. H. -E., 1,000

inner and the outer layers whose connections are less firm than those among the cells /Figs 3 and 4/. Molnár /1979/ claimed that Sphaerospora carassii is less pathogenic than implied from the intensity of infections because the infected stratified epithelium serves only as a supporting tissue while the secondary lamellae do not become affected by the parasite. Our findings provide evidence that the gas exchange surface of secondary lamellae is reduced which necessarily impairs the respiration in serious infections with Sphaerospora sp. The branchial lesions are important enough to consider Sphaerospora sp. a serious pathogen even without intervention of concurrent infections.

In one stock of second year carps, we recorded a unique occurrence of Sphaerospora sp. in the blood of a specimen suffering from gill sphaerosporosis. The blood contained numerous mature spores, sporoblasts and plasmodia of early developmental stages.

2. Hepatic chloromyxosis

Chloromyxum cyprini Fujita, 1927 was found in gall bladders of carp and grass carp fingerlings from different localities. In six out of ten grass carp specimens examined, numerous plasmodia of the same species were present in the liver parenchyma. Some plasmodia were sharply outlined and scattered in the parenchyma /Fig. 5/; the others, with cell surface less distinctly seen contained mature spores and formed numerous aggregations /Fig. 6/. In the material we could examine, the principal pathologic change was massive necrosis of hepatic parenchyma. The regeneration of hepatic cells and reactive inflammation which usually follows necrosis were poorly expressed. There were only small groups of inflammatory cells and proliferating fibroblasts around the necrotic foci. The extent of necrosis indicated a poor prognosis. The formation of mature spores in the plasmodia localized in liver parenchyma gives evidence that Chloromyxum cyprini, considered until now to be a strictly coelozoic species, can also invade hepatic tissue. The changes in carp fingerling liver, although less extensive than in grass carps, had essentially the same character.

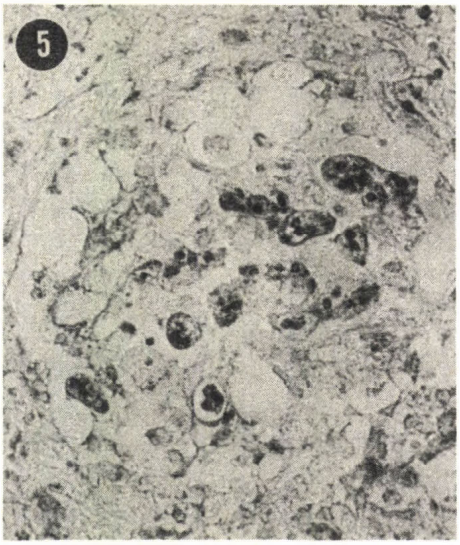

Figure 5 Chloromyxum cyprini. Necrotic liver parenchyma with a group of plasmodia. H.-E., x 550

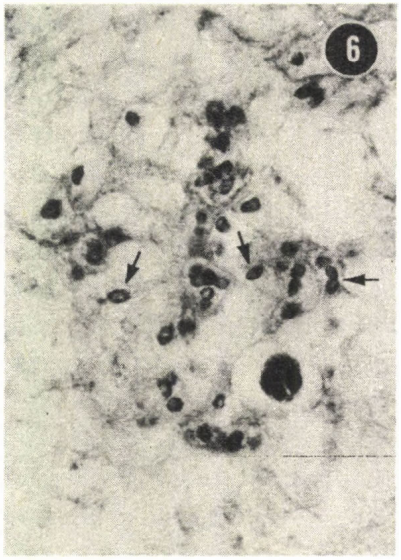

Figure 6 Chloromyxum cyprini. A group of plasmodia with fuzzy outlines and generative cells /arrows/; bottom right, a single mature spore in the necrotic liver parenchyma. Giemsa, x 1,100

3. Renal myxozooses of carps

Large scale examination of carp fingerlings and second year carps from the region of Southern Bohemia disclosed four widely distributed, kidney infecting myxozoan species; i.e., Mitraspora cyprini Fujita, 1912, Sphaerospora sp. different from that found in gills, Myxobolus cyprini Doflein, 1898 and developmental stages identified tentatively as Hoferellus cyprini Doflein,1898. There has been no previous record of the first two species in Czechoslovakia, while Hoferellus was found only very rarely. These infections are rather extensive; out of 23 localities examined, Sphaerospora was found in 15 /=65 %/, Mitraspora in 5 /=21 %/, Hoferellus in 4 /=17 %/, and Myxobolus in 8 /=34 %/. It is rather surprising that these infections have passed unnoticed until now. In some carps the kidney harbours only a single species while mixed infections are common in Mitraspora and Sphaerospora. Our data on pathogenesis of renal myxozoa are based on follow-up studies of selected carp populations from different ponds.

Quite often, serious infections with renal myxozoans were indicated by slight enlargement of trunk kidney, the edges of which were noticeably rounded. To an experienced eye, these changes were easy to detect.

Mitraspora cyprini and Sphaerospora sp. are found in the kidney tubuli. The former has plasmodial stages up to about 60 µm in size, producing many spores the structure of which corresponds to a certain degree to previous descriptions /Fujita 1912, Ahmed 1973, Alvarez Pellitero et al. 1979/. Sphaerospora sp. forms round plasmodia /Figs 7 and 8/ up to only 10 µm, producing two spores each. Unlike spores of S. angulata Fujita, 1912 reported from Hungary by Molnár /1980/ spores of Sphaerospora sp. are globular shaped in fresh state like that of S. carassii Kudo, 1919, with both polar capsules of equal size /Fig. 9/. Mature spores were found only in spring from March to June.

Renal lesions associated with Mitraspora cyprini and Sphaerospora sp. infections differ in details only, most of the changes are common to both. Histologic appearance varies with the stage of development and with the intensity of infection. The earliest histological alterations in Mitraspora and Sphaero-

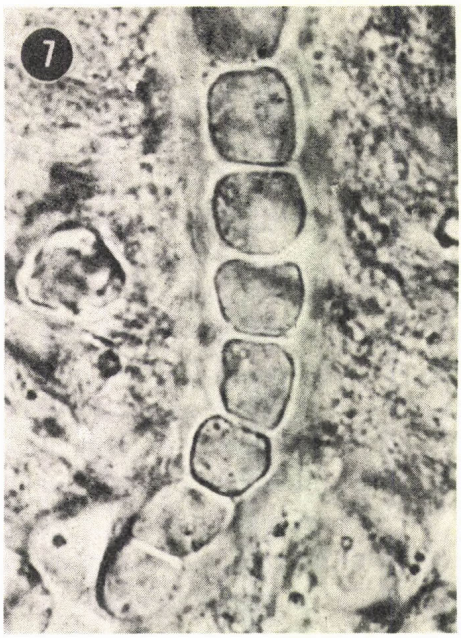

Figure 7 Chain of plasmodia of Sphaerospora sp. filling the tubular lumen. Squash preparation of fresh tissue. x 1,300

Figure 8 Plasmodia of Sphaerospora sp. in the tubular lumen. Squash preparation. x 900

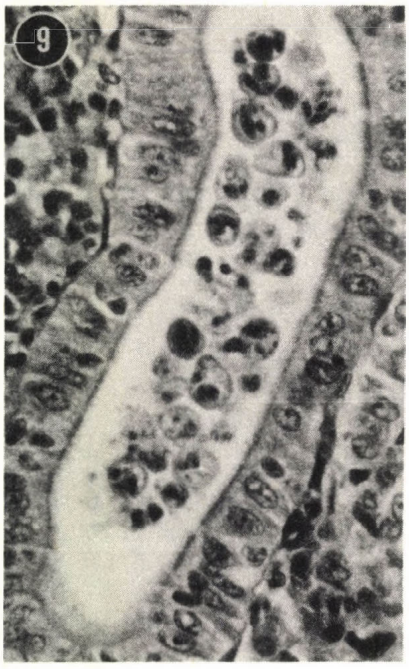

Figure 9 Mature spores of Sphaeromyxa sp. in the tubular lumen. Squash preparation. x 1,700

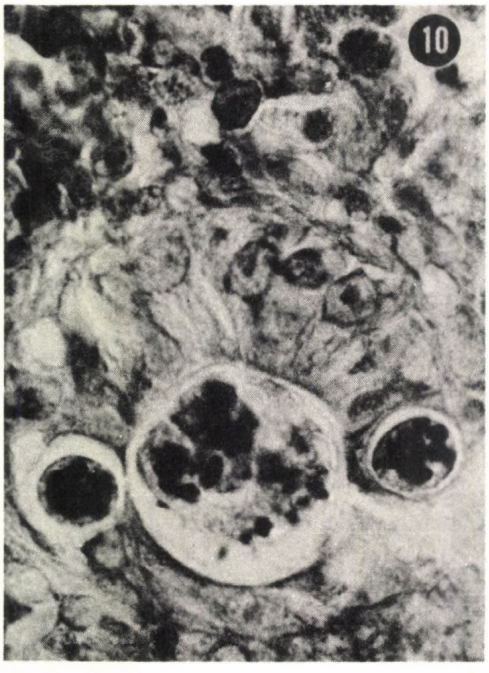

Figure 10 Mitraspora cyprini: plasmodium filling the tubular lumen and two smaller ones located intracellularly. Giemsa, x 1,100

spora infections were found in the tubular epithelium, where both species develop intracellularly. In Mitraspora, intracellular development /Fig. 10/ was first observed by Ahmed in 1973; in Sphaerospora, ours is the first record /Fig. 11/. Intracellular developmental stages cause displacement of nuclei, cytoplasm being reduced to a thin layer around the parasite. Ultimately, infected epithelial cells are destroyed, releasing the parasite into the tubular lumen. If the tubular epithelial lining is not completely destroyed, the persisting uninfected cells are displaced or disarranged. This is more pronounced in Mitraspora infections, probably because of the larger size of developmental stages. A few times we have found plasmodium of Mitraspora with mature spores still in the epithelial lining, while as a rule spores are formed only in plasmodia within the tubular lumina. In both Mitraspora and Sphaerospora the masses of plasmodia usually completely fill the tubular lumina. Such accumulations in the lumina cause dystrophic changes manifested by distinctive but not pathognomonic hyaline droplet degeneration and vacuolation. In massive infections, tubular epithelium is subject to more pronounced regressive changes such as flattening of the epithelial cells with disappearance of their brush border and atrophy by fibrosis in advanced cases.

We have tentatively identified the vegetative myxozoan stages as Hoferellus cyprini found intracellularly in the epithelial lining of kidney tubules. They are globular, containing 1 to 9 generative cells and 1 to 6 vegetative nuclei; there were no sporoblasts present.

The appearance of infected, disfigured tubuli is identical with Plehn's /1924/ picture and description of H. cyprini infection in carps. That is why we have identified our findings as H. cyprini. We have never encountered mature spores and thus, the identification is still only tentative. There is, however, no positive evidence that anybody else did find mature spores belonging to the intracellular vegetative stages. In addition to Mercier's /1908/ oversimplified drawing of mature spore of Hoferellus cyprini, which strongly resembled the spore of Mitraspora, the only existing drawing of mature spores is the original one of Doflein /1898/ subsequently reproduced in the past and present by all textbooks on fish parasitology.

Figure 11 Sphaerospora sp. Plasmodia - some well preserved, some disintegrated - in the tubular lumen. Small intracellular stages indicated by arrows. H.-E., x 900

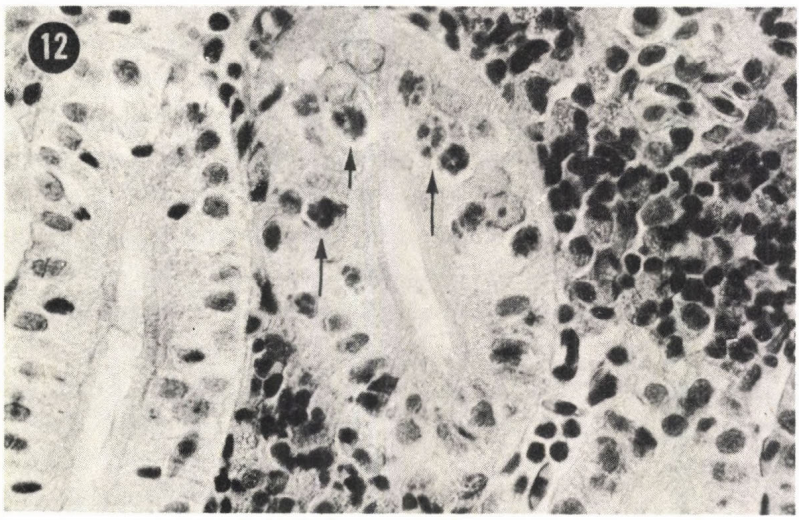

Figure 12 Hoferellus cyprini. Early developmental stages located intracellularly /arrows/. H.-E. x 300

Doflein's drawing of Hoferellus spore found allegedly in plasmodia passed into the tubular lumen is also simple and diagrammatic but to an extent it raises suspicion that he might actually see spores of Mitraspora cyprini. Mitraspora spores are quite similar /the caudal bristles are not always clearly seen/ and occur within plasmodia in the tubular lumen. Molnár /1979/, who encountered aggregations of intracellular developmental stages, similar to what we consider to be Hoferellus, took them for cysts and postulated that the organisms probably represented developing stages of Myxobolus cyprini. We have also observed spores of Myxobolus cyprini in close association with Hoferellus stages without, however, any safe evidence in favour of their common identity.

Though the causative agent has not been determined satisfactorily, the histopathological changes provoked are worth recording. Authors who reported on the occurrence of H. cyprini failed to describe associated histopathological changes which are quite characteristic and unlike changes in any other myxozoosis.

The lesions start to unfold when the first few developmental stages enter the host cells. Isolated infected cells manifest relatively small, sometimes undetectable increase in size /Fig. 12/. As seen by electron microscopy, there is no parasitophorous vacuole around the Hoferellus stages. Growth and multiplication of the developmental stages within the epithelial cells change the appearance of the tubular epithelial lining. Massive distention and sometimes monstrous increase in the size of the individual nuclei cause thickening of the tubular wall and stenosis of the tubular lumen. As seen in tubuli cut longitudinally, relatively long stretches of their course are affected. Even in more advanced stages when the diameter of tubuli and the epithelial lining of the affected tubulus is repleted with developmental stages, the brush border persists undamaged /Figs 13 and 14/. Histological examination of about 97 cases has proved that changes occur exclusively in convoluted tubuli, never in collecting ducts and mesonephric duct easily discernible by their connective tissue coat. These changes are quite characteristic and prevail for several months. As long as the

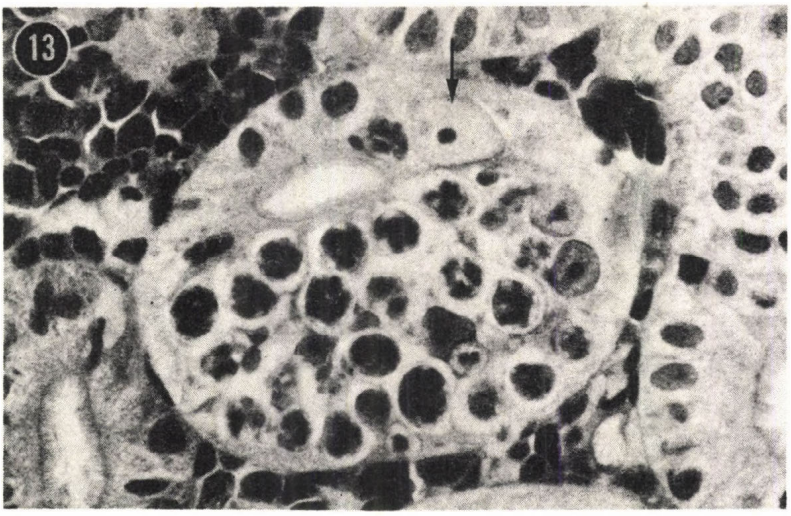

Figure 13 More advanced stage of development of H. cyprini. Note the hypertrophic nuclei of host cells /arrow/. H.-E. x 1,200

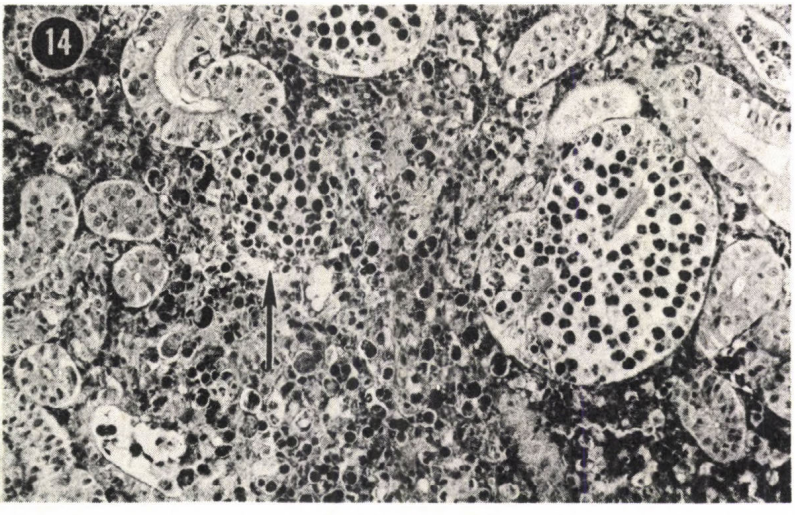

Figure 14 Developmental stages of H. cyprini fill the enlarged tubule with still preserved basal membrane and brush border; note the stenosis of the lumen. Arrow points at a mass of developmental stages released from another tubule surrounded by granulation tissue. H.-E., x 300

parasite development is confined to the tubulus with well preserved basement mambrane there are no lesions associated. Once the base membrane ruptures, release of the parasite stages into the renal interstitium provokes a granulomatous inflammatory reaction. Initially, the inflammatory response is manifested predominantly by the appearance of macrophages, fibroblasts, some lymphocytes and newly formed capillaries /Fig. 14/. It is followed later by the formation of various sized granulomas with a necrotic centre surrounded peripherally by fibrocytes /Fig. 15/. The rupture of the base membrane is the common fate of tubular lesions. Parasite stages could only exceptionally be observed in the tubular lumen and then they were in the state of degeneration; sometimes cast-like contents were present, but never mature spores. Renal lesions due to Hoferellus are clearly progressive, developing over the span of several months. The final damage to kidneys may be quite considerable, resulting in diseased condition and losses in the affected stocks. Sometimes losses up to 30 % were incurred.

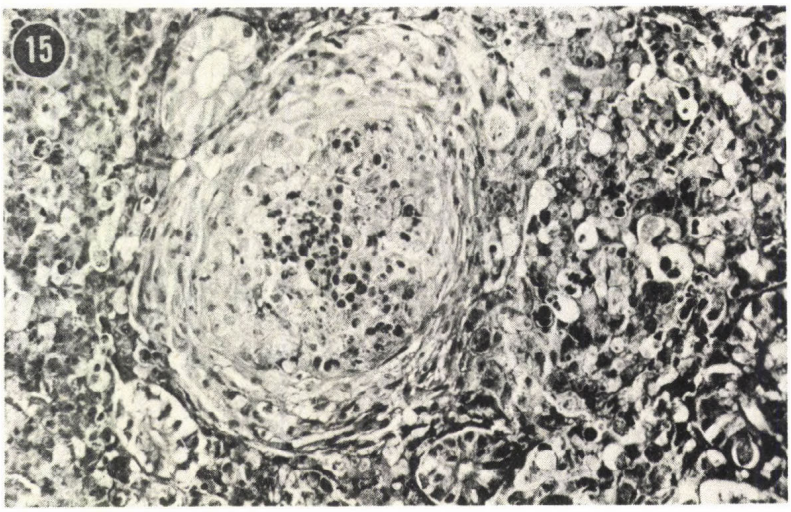

Figure 15 Granulomatous inflammatory reaction due to released developmental stages of H. cyprini. H.-E., x 300

Myxobolus cyprini has been found in carps in 6 of 18 localities. Its presence was only signalled by findings of typical spores; dispersed developmental stages and sporogenesis are difficult to differentiate. The spores are of highly variable shape having or lacking a peculiar semicircular thickening on the posterior border, with, however, characteristic polar capsules containing 3 to 4 slanted windings of the polar filament. Myxobolus cyprini spores were found regularly in melano-macrophage centres, and quite loosely distributed in renal interstitium, rarely in the walls of mesonephric duct and exceptionally in the lumen of renal tubuli. In addition to kidneys, they were found in gills, intestine and other organs. It is interesting that mature spores were found in the foci of granulomatous inflammatory reaction resulting as a response to developmental stages of Hoferellus released from the tubular epithelium. However, we failed to find any pathological changes in the kidney which could be linked to the development of Myxobolus cyprini.

DISCUSSION

The aim of this communication was to direct attention to the problems that may be presently caused in carp culture by parasitic protozoa. The above findings indicate a wide distribution of renal myxozoans in carp fingerlings in Czechoslovakia, which can be compared only with extensive infection of ectoparasitic protozoa or with the commonly found unidentified blood protozoan described by Csaba /1976/. Mitraspora cyprini and Sphaerospora sp. have not been reported so far in our country; did the previous investigations simply fail to detect them or have they been introduced recently? Lately, both species were reported for the first time from goldfish in the U.S. /Hoffman and Mitchell 1980/. It may well be possible that they have persisted in Czechoslovakian fish ponds for a long time at a low level of infections, while in the high-rate stocked ponds they have recently started to flourish. While the distribution of blood flagellates can be quite limited by the eradication of leeches, directly transmitted myxozoans may benefit from densely stocked ponds.

Although carp has been the subject of ichthyoparasitological research for almost a century, studies of its protozoan faunule continues to yield interesting data on the life cycle of its myxozoan parasites. Findings of mature spores in the blood may not be so exceptional as the survey of existing literature might indicate.

Further research has to clarify whether the occurrence of the developmental stages of gill Sphaerospora in the blood indicates simply the rupture of damaged blood vessels or in serious infections the parasite proceeds to the bloodstream as to another site of development.

Extremely small numbers of myxozoans with known intracellular stages in their life cycle have been recorded. It is startling that in myxozoa infecting carp kidney alone, the intracellular stages occur in three species!

Our data on Hoferellus cyprini are in a way preliminary, since we have failed to detect mature spores. Vegetative stages gradually proliferating without any signs of sporogony for such a long period of time /in one case we followed it for 8 months!/ are quite unique among both coelozoic and histozoic myxozoa.

Another important point is the disclosure of the pathogenic potential of the carp myxozoa in question. Species of the genus Chloromyxum, C. truttae Léger, 1906 and C. coregoni Bauer, 1958 are known to cause hypertrophy of the gall bladder, jaundice and intestinal disorders /Schulman 1966/ but thus far no positive proof of liver tissue infection has been supplied.

Sphaerospora sp. from the gills of carps, if present in an infection, is a primary pathogen reducing the respiratory area of secondary lamellae and disrupting their structure. It can endanger its host even without the synergic action of other pathogens.

Pathogenic potential of kidney infecting species of the genus Sphaerospora has already been known. Lethal sphaerosporosis of head kidney in tenches, caused by Sphaerospora pernicialis was recorded by Léger /1930/. Pathological changes due to renal sphaerosporosis and mitrasporosis in goldfish were mentioned by Ahmed /1973/ and by Hoffman and Mitchell /1980/. In carps, Molnár /1980/ did not ascribe a pronounced pathogenic action to Sphaerospora angulata which infect carp renal tubules.

Our results confirm the pathogenic action of both Sphaerospora and Mitraspora, which along with Hoferellus can impair the health of carp fingerlings, stage most important in rearing carps.

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THE EFFECT OF BOTHRIOCEPHALUS ACHEILOGNATHI INFECTION ON THE PROTEASE AND α-AMYLASE ACTIVITY IN THE GUT OF CARP FRY

I. MATSKÁSI

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

ABSTRACT

Common carp fry infected by the tapeworm, Bothriocephalus acheilognathi Yamaguti, 1934, showed significantly lower intestinal trypsin and chymotrypsin activities than non-infected fry reared in the same pond farm. Extracts prepared from larval and adult stages of the tapeworm depressed trypsin and chymotrypsin activities of fish gut washing fluids "in vitro". The infection had no effect on α-amylase activity of fish gut.

INTRODUCTION

Infection by Bothriocephalus acheilognathi has been found to considerably depress the growth and development of fry. Worms adhering to the host's gut wall injure the lining epithelium, produce and secrete toxic materials, and obstruct the passage of intestinal contents mechanically, by their bulk. Furthermore, certain, not clearly understood influences of the parasite have been supposed to play a role in the pathogenesis of bothriocephalosis /Bauer et al. 1977/.

Physiological investigations into the function of digestive enzymes associated with the gut of fishes disclosed that parasite infection depressed the host's intestinal protease activity /Braun et al. 1968/. Intestinal trypsin and α-amylase activity was lower in Coregonus lavaretus host's infected by the tapeworm Proteocephalus longicollis than in non-infected ones. In vitro experiments have shown that living parasites depressed intestinal trypsin activity to a greater degree than parasite extracts

added to gut extract /Reichenbach-Klinke and Reichenbach-Klinke 1970/. Mammalian trypsin and α - and β -chymotrypsin, added to the maintenance medium of Hymenolepis diminuta, were irreversibly inactivated by the parasite /Pappas and Read 1972 a, b/. Inhibitors inactivating mammalian trypsin and chymotrypsin were demonstrated in the maintenance medium of Ligula intestinalis plerocercoid larvae collected from bream /Abramis brama/, and in extracts of homogenated larval and adult specimens as well /Matskási and Juhász 1977/. The inhibitors could be separated from plerocercoidal somatic extracts by gel chromatography and their molecular weights were estimated /Matskási and Németh 1977/.

Investigations into the influence of Bothriocephalus acheilognathi infection on trypsin, chymotrypsin and α-amylase activities in the gut of the carp fry are reported in this paper.

MATERIAL AND METHODS

Carp fry collected in pond farms in Hungary were used in the experiment. Enzyme activities measured in the gut of the infected fish were always compared to the similar activities determined in non-infected controls taken from the same habitat. With regard to seasonal fluctuations in the intensity of infection, protease activity determinations were carried out in one experimental and one control group each in spring and summer, and in two groups each in the autumn. Experimental and control fry were exterminated simultaneously after the last feed intake in each test series. Gut was removed from the dissected fish, ligated at both ends, 0.5 ml distilled water was injected into it /the mean wet weight of the gut was 0.5 g/. Ten min. later the rinsing fluid was recovered by syringe and centrifuged at 19,500 g for 30 min. The supernatant was used for enzyme assays. Guts were homogenated in 10 volumes of distilled water, and the homogenates centrifuged at 19,500 g for 60 min at +5 °C. The same procedure was employed for preparation of extracts from adult tapeworms.

Trypsin activity was determined on N-α-benzoyl-arginine--ethyl ester /BAEE/ substrate, as proposed by Schwert and Takenaka /1955/, chymotrypsin activity on N-benzoyl-L-tyrosine-ethyl ester /BTEE/ substrate, according to the method of Hummel /1959/, in a system described in details in a previous publication /Mats-

kási and Juhász 1977/. Unit protease activity was defined as the hydrolytic activity producing increase of the optical density /OD/ by 1,000 OD unit in one min, at 253 and 256 nm on BAEE and BTEE substrates, respectively. The activity of α-amylase was measured using the method of Ujihara et al. /1965/.

Enzyme activity differences between the infected and non-infected groups were evaluated by Student's "t" test. The variation coefficient was determined for 20 routine measurements in each series, to assess reproducibility; the values of 11.8 and 15 % were obtained for trypsin and chymotrypsin activities, respectively.

RESULTS

Trypsin and chymotrypsin activities determined in the intestinal washing fluids of carp fry infected and not infected with Bothriocephalus acheilognathi are shown in Table 1. Despite the considerable individual variations, the group average for trypsin activity was lower in each infected group relative to that found in the controls. Chymotrypsin activity fell significantly below the control level in the fry tested in April and October while in those tested in August and November the probability level was above 5 %, although there was a demonstrable activity decrease relative to the control in both instances.

Data on the influence of tapeworm extract on the trypsin and chymotrypsin activities of intestinal washing fluid are shown in Table 2. In this series, activity determinations were carried out in the following system: 2.3 ml 0.1 M Tris HCl buffer /pH 7.4/, with 8 mM $CaCl_2$ added + 0.5 ml substrate + 0.1 ml intestinal washing fluid + 0.1 ml parasite extract. Determinations were carried out with gut-washing fluid of non-infected fry, collected in April.

No linear relationship could be demonstrated between the intensity of infection /i.e. total parasite weight/ and drop of gut protease activity.

Protease activity determinations in intestinal rinsing fluids were followed by similar assays in the gut wall extracts. Unlike rinsing fluids, neither trypsin nor chymotrypsin activity differed between extracts prepared from the guts of infected and non-infected fry /Table 3/.

The B. acheilognathi infection had no demonstrable effect on the α -amylase activity of intestinal washing fluid.

Table 1 Results of the analysis of proteolytic enzyme activities /U/ml/

	12th April				15th August			
	Trypsin		Chymotrypsin		Trypsin		Chymotrypsin	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
n	10	10	10	10	11	16	11	16
$\bar{x}$	0.232	0.005	0.500	0.320	0.185	0.081	0.524	0.319
S_D	0.054	0.007	0.109	0.084	0.117	0.117	0.411	0.427
P %	< 0.1		< 0.1		< 5		< 15	
	20th October				10th November			
n	6	10	6	10	7	5	7	5
$\bar{x}$	0.435	0.221	0.107	0.043	0.211	0.060	0.141	0.076
S_D	0.144	0.053	0.061	0.031	0.263	0.038	0.104	0.051
P %	< 0.1		< 1		< 5		< 10	

Table 2 Effect of worm extract on the proteases of fish gut /U/ml/

	Gut washing fluid			Gut washing fluid + worm extract			Significance P
	$\bar{x}$	SD	n	$\bar{x}$	SD	n	
BAEE-splitting activity /trypsin/	2.72	0.46	10	0.21*	0.13	10	< 0.001
BTEE-splitting activity /chymotrypsin/	2.09	0.31	10	0.14**	0.09	10	< 0.001

* Activity was determined after 5-minute preincubation

** Activity was determined after 15-minute preincubation

Table 3 Protease activities of gut wall extracts /U/ml/

	Infected $\bar{x}$	SD	n	Non-infected $\bar{x}$	SD	n	Significance P
BAEE-splitting activity	3.14	0.65	10	3.18	0.88	10	< 0.05
BTEE-splitting activity	2.72	0.57	10	3.13	0.79	10	< 0.05

DISCUSSION

Formerly only a trypsin-like protease had been detected in the fish gut /Philips 1969, Barrington 1957/, but later testing in strong alkaline medium /optimal pH range: 10.0 - 11.0/ revealed the presence of certain not clearly identified protease in the gut of common carp /Cyprinus carpio L./ and silver carp /Hypophthalmichthys molitrix/ /Onishi et al. 1973 a, Rágyánszki and Jónás 1977/. The activity of the digestive enzymes, including protease, depends on many factors in fishes. Secretion and function of such enzymes are considerably influenced by the temperature and pH of the water habitat, seasonal fluctuations in the external environment, length of time between feed intakes and quality of feed /Phillips 1969, Reichenbach-Klinke and Reichenbach-Klinke 1969, Onishi et al. 1973 a, b/. Activity was also found to differ between given intestinal segments /Braun et al. 1968, Rágyánszki and Jónás 1977/. Variations due to these factors were eliminated in the present study by comparing the gut enzyme activities measured in infected fishes to those found in contemporary non-infected fry hatched and kept under identical conditions in the same pond, and killed simultaneously with the infected ones for experimental purposes. In every case, the entire gut was rinsed and extracted for enzyme assay, since tapeworm specimens can also occur in the distal gut segment, if the infection is serious.

In vitro evidence of protease inactivation by living intact tapeworms, and by inhibitors secreted by the latter, has been presented earlier /Pappas and Read 1973 a, b, Matskási and Juhász 1977/. Bothriocephalus acheilognathi was not previously studied in this respect. In the present experiments the somatic extracts of B. acheilognathi inactivated the trypsin and chymo-

trypsin activities of fish gut rinsing fluids in vitro. From this it may be concluded that the tapeworms parasitic in the intestine of fishes depress the host's proteolytic enzymes also in vivo, above all through the production and secretion of specific inhibitors.

Unlike the statement of Reichenbach-Klinke /1969/ and Reichenbach-Klinke /1970/, we failed to find lower activity of α-amylase in the gut of infected carp fry.

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TWO FISH PATHOGENS, PARVICAPSULA SP. AND MITRASPORA CYPRINI (MYXOSPOREA) NEW TO NORTH AMERICA

G.L. HOFFMAN

U.S. Fish and Wildlife Service, Fish Farming Experimental Station, Stuttgart, Arkansas 72160, USA

ABSTRACT

Two fish diseases, new to North America, are reported. Proliferative chronic nephritis of pen-reared coho salmon, Oncorhynchus kisutch, caused by Parvicapsula sp., and kidney bloater disease of goldfish, Carassius auratus, caused by Mitraspora cyprini are described.

INTRODUCTION

In the past three years two new parasitic diseases have appeared in American cultured fishes. Parvicapsula sp./Myxosporea/, an unusual undescribed parasite /variously nick-named schomoo, Casper Ghost, kitty cat/ of pen reared coho salmon /Oncorhynchus kisutch/ causes chronic proliferative nephritis and apparent significant mortality. In 1979 it was discovered as an unidentified causal agent by Dr. Lee Harrell, NMFS Aquaculture Station, Manchester, Washington; Dr. Marsha Landolt, College of Fisheries, University of Washington, Seattle, Washington; Paul Wagner, 510 Washington Ave., Bremerton, Washington; and Dr. Wm. Yasutake, National Fisheries Research Center, Seattle, Washington /listed alphabetically/. Specimens were sent to me as well as others. Subsequently, the generic identification was made independently by Drs. Leo Margolis and Bob Kabata, Pacific Biological Station, Nanaimo, B.C., Canada and Dr. Victor Sprague, Chesapeake Biological Laboratory, University of Maryland, Solomons, Maryland. Verification of the genus of this unusual pathogen was made by the original describer of the genus, Dr. S.S. Shulman, Academy of

Sciences, Leningrad, USSR. All of the above have contributed to this preliminary study.

The other pathogen, Mitraspora cyprini /Myxosporea/, the goldfish kidney bloater, is a recent import to the United States, having come here with goldfish shipments. It invades the kidney, causing massive hyperplasia and usually death of goldfish. This also is considered a preliminary report.

For taxonomic relationships of these two myxosporidans to others I have consulted Shulman /1966/ and Kudo and Meglitsch /1981/.

MATERIAL AND METHODS

1. Parvicapsula sp. Moribund pen-reared coho salmon /Oncorhynchus kisutch/ were obtained from two aquaculture establishments of the Pacific Northwest Coast. Wet squashes were prepared from formalin-preserved fish. Histological sections were stained with hematoxylin and eosin , and Giemsa's.

2. Mitraspora cyprini. Moribund goldfish /Carassius auratus/ were obtained from a goldfish farm in the Southeastern United States. Infected kidney material was studied in fresh wet squashes and sectioned formalin-fixed kidney.

RESULTS

1. Parvicapsula sp.

This previously undescribed species is found in the kidney of pen-reared coho salmon /Oncorhynchus kisutch/. Development and mature forms are present in the epithelium of the renal tubules, but the possibility of intercellularity was not determined. Plasmodia seen are 6 to 16 µm in diameter. One mature sporoplast, 16 µm in diameter, contained 4 spores. Many mature spores are present in the lumina of the renal tubules /Fig. 1/.

The spores are elongate, usually curved but variable /Fig. 2/. One valve appears to be larger, the other smaller and flattened. Suture line hardly discernible; it runs from one end to the other, passing between the polar capsules. Very small spherical polar capsules at the anterior end look like eyes and are enclosed by the head-like vestige of the capsuligenic cells. The head organ is usually slightly raised unilaterally so that the

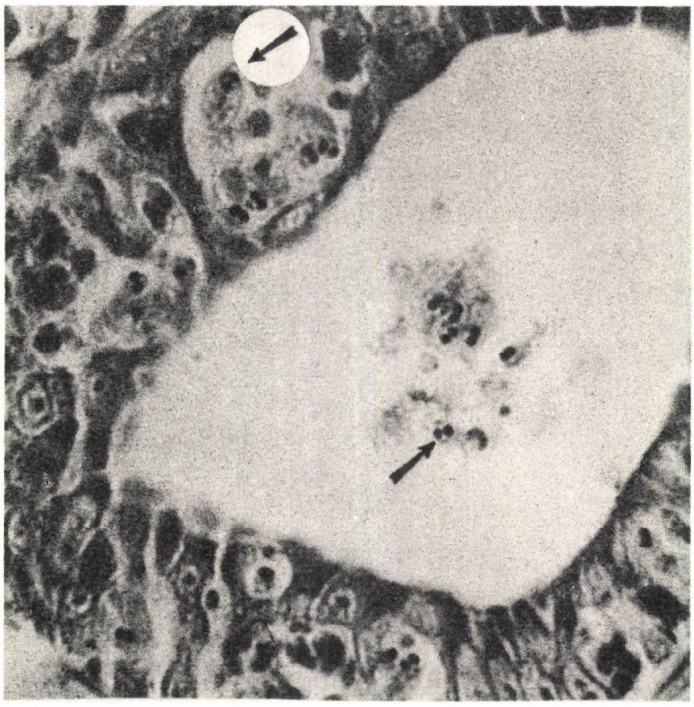

Figure 1 Parvicapsula sp. /arrows/ in tubule epithelium and lumen of Oncorhynchus kisutch kidney. Polar capsules stained dark. Giemsa stain. x 400

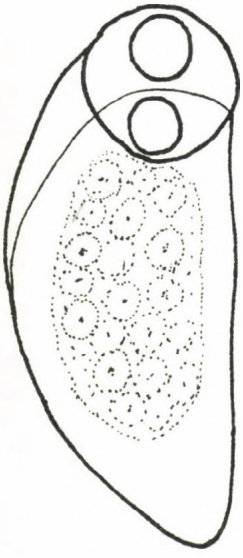

Figure 2 Parvicapsula sp., most usual shape, drawn to scale, showing suture line, sporoplasm, and two spherical polar capsules in "head" organ

polar capsules lie in the vertical plane. The sporoplasm does not fill the entire cavity of the spore; nuclear details have not been determined.

This new species most closely resembles P. asymmetrica Shulman 1953 but is smaller, more curved, and the capsulogenic cells more prominent, giving it the "head" appearance. The polar capsules are smaller and spherical and lie in a plane perpendicular to the sutural line. Four other species of Parvicapsula have been described: P. asymmetrica Shulman, 1953, P. shulmani Kovaleva and Gaewskaja 1951, and P. lobata /Syn. Ceratomyxa lobata /Evdokimova 1977/, the latter according to S. Shulman /pers. comm./. The possible relationship of Conispora renalis /Sankaruthri, 1977/ should be investigated.

2. Mitraspora cyprini Fujita 1972

The life cycle and pathogenicity of this parasite of goldfish /Carassius auratus/ and carp /Cyprinus carpio/ have been described by Ahmed /1973, 1974/; previous work was published by Fujita /1912/, Kudo /1920/ and Hoshina /1968/, all from Japanese material. According to Ahmed /1973, 1974/ the spores are ingested, the sporoplasm leaves the spore, penetrates the intestinal mucosa and is probably circulated by the cardiovascular system, emerging in the kidney. The epithelial cells of the renal tubules are penetrated with ensuing growth and multiplication before spores are produced. During the process, the kidneys /sometimes unilateral infection/ become tremendously enlarged. My material appears to be exactly like Ahmed's /Figs 3-6/ except that I did not recover mature spores. Histopathology reveals nonneoplastic papillary cystic hyperplasia /Harshbarger, pers. comm., Smithsonian Inst., Washington, D.C./. The infection is seasonal with spores being produced in late winter. On two occasions all of my infected fish died before spore maturation although I did see one immature spore in January. When infected and uninfected fish were held in aquaria, October-May 1978-1979, none of the accompanying uninfected ones became infected. Presumably the life cycle can be completed only in natural or simulated ponds, as is the case with whirling disease /Myxosoma cerebralis/ of trout /Hoffman 1976/. This is the first record of this parasite in North America.

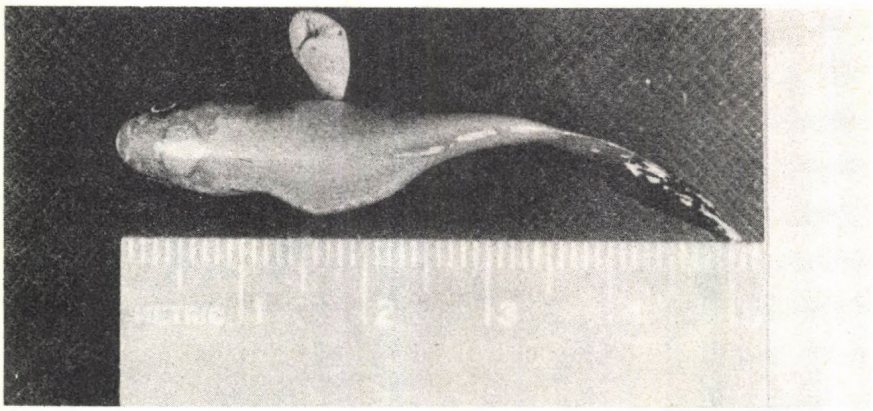

Figure 3 Mitraspora cyprini, unilateral infection in kidney of Carassius auratus

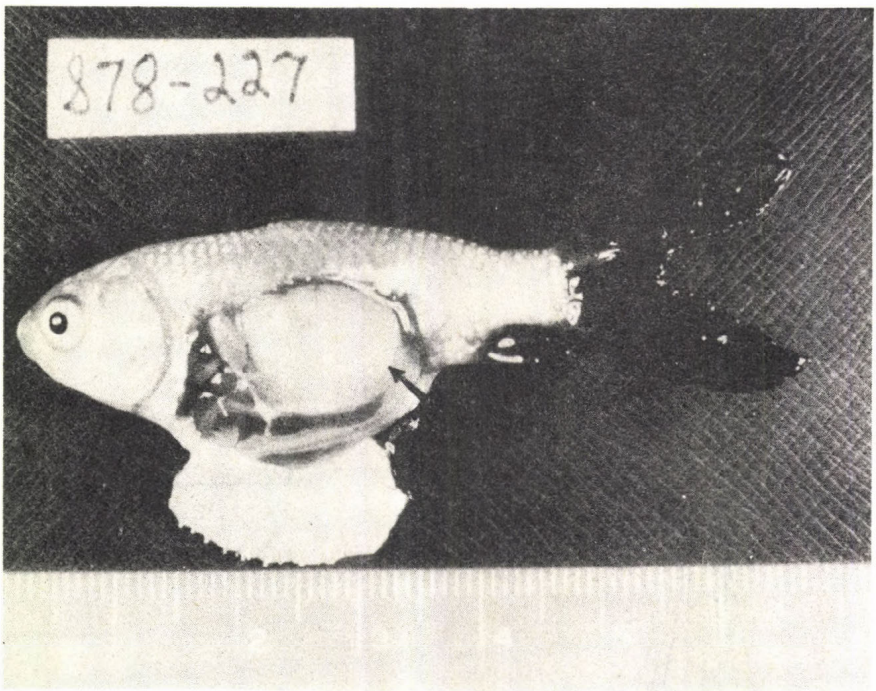

Figure 4 Carassius auratus infected with Mitraspora cyprini; opened to show enlarged kidney

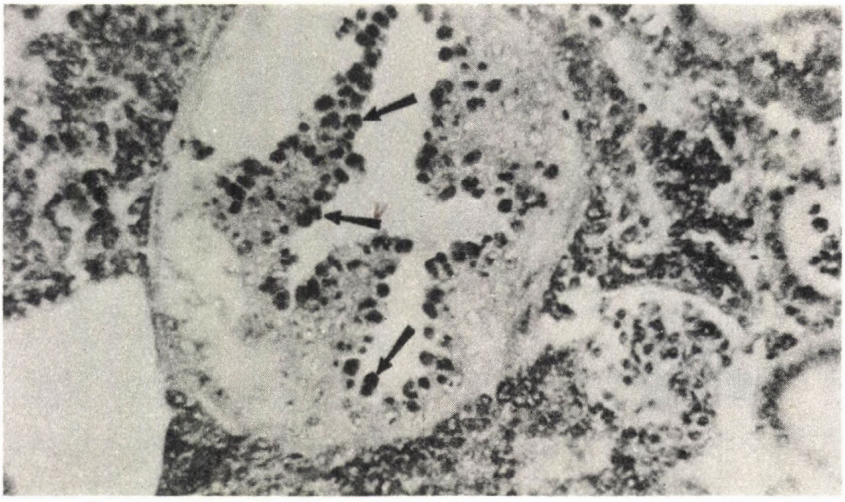

Figure 5 Mitraspora cyprini. Section showing parasites /arrows/ in renal tubule and hyperplastic pathology. Giemsa stain. x 160

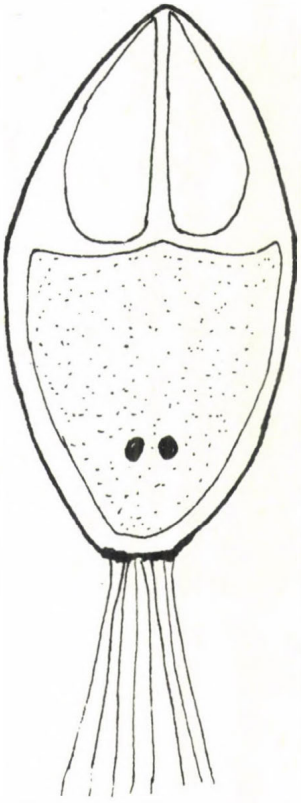

Figure 6 Mitraspora cyprini spore. Free hand sketch based on published reports

DISCUSSION

This new Parvicapsula sp. is unique among the four known species, P. asymmetrica Shulman, 1953; P. shulmani Kovaleva and Gaewskaja, 1951; P. unicornis Kabata, 1962; P. lobata /Evdokimova 1977/ in that it is not found in a bottom dwelling fish. P. asymmetrica is found in the urinary bladder of Cyclopterus lumpus, White Sea, P. shulmani is found; P. unicornis is found in the urinary bladder of Callionymus lyra, Limanda limanda and Lepidorhombus whiffiagonis, North Sea, Atlanic Ocean; P. lobata is found in the urinary bladder of Austroatherina incisa, Patagonian Shelf, Atlanic Ocean. It is further unusual in that it is found as a histozoic parasite in the renal tubules, whereas the other species were found in the urinary bladder. In this respect its life cycle resembles that of Mitraspora cyprini although the kidneys do not become as enlarged. More details on the pathology will be published later, but most of the damage appears to be destruction of renal tubule epithelium /proliferative kidney disease Fig. 1/. Apparently the spores accumulate in the vicinity of the pens /cages/ and although the intensity of infection becomes very great, the mortality is a low grade type. Total losses, however, are estimated to approach 30 %. There is a possibility that this Parvicapsula is found in other fish, and it may be that the coho salmon is not the primary host in more natural surroundings. A search for other hosts should be made.

Control measures for other myxosporeans in pond culture include pond drainage, drying and/or application of lime or calcium cyanamide. I am aware of no reported method to treat such a situation in ocean pens. Chemotherapy is unknown for Parvicapsula and, if effective, would have to be administered repeatedly to contact trophozoites which are probably produced over a long period of time. Nitrofurazone, Furazolidone, sulfaisomidine, sulfamonomethoxine, sulfadimethoxine and Amprolium apparently suppress sporozoans of fish /Awakura and Kurahashi 1967; Taylor et al. 1973; Nagel 1977/, but their effectiveness for Parvicapsula has not been demonstrated.

According to goldfish producers in the United States and fish pathologists in Japan, Mitraspora cyprini comes and goes for unknown reasons. One year there may be many infected fish in a pond but sometimes very few can be found the following year. The clinical signs are almost pathognomonic but sometimes could be confused with bacterial dropsy. However, if only one kidney is infected /Fig. 3/, I believe the signs are pathognomonic. Presumptive diagnosis can be made on the signs and presence of trophozoites in the kidney tubules. Further verification should be sought in late winter, when typical spores are produced.

It is not known when Mitraspora cyprini came to North America. I found it for certain in 1979 and was told that the affected farm had received recent shipments from Japan. Until more is learned, one might employ control measures such as those used for whirling disease of trout /Hoffman 1976/.

CONCLUSIONS

Parvicapsula sp. causing proliferative kidney disease of pen-raised coho salmon, is new to science but most closely related to P. asymmetrica found in the urinary bladder of the bottom fish, Cyclopterus lumpus. The life cycle in, and out, of the fish has not been studied thoroughly-until that is done, no definitive control measures can be recommended.

Mitraspora cyprini, causing spectacular neoplastic papillary cystic hyperplasia of goldfish, is reported for the first time in the United States. Because the disease is proliferative, and fatal, this parasite is considered dangerous to the goldfish industry.

It is important that proper diagnosis of both of these diseases be made to insure against shipping them inadvertently to locations where they don't already exist.

ACKNOWLEDGEMENTS

The following have helped, one way or another, during these studies /listed alphabetically/: L. Harell, NMFS, Manchester, Washington; J. Harshbarger, Smithsonian Inst., Washington, D.C.; Z. Kabata, Pacific Biologic Station, Nanaimo, B.C., Canada; M. Landolt, Univ. Washington, Seattle; F. Leteux, Stoutland, Missouri;

L. Margolis /address as Kabata/; S. Newman, Bio Med., Seattle, Washington; T. Otto, Rangen Research, Hagerman, Idaho; S.S. Shulman, Academy of Sciences, Leningrad, USSR; C. Smith, Bozeman Dev. Center, Bozeman, Montana; V. Sprague, University of Maryland, Solomons, Maryland; P. Wagner, Bremerton, Washington; W. Yasutake, National Research Center, Seattle, Washington.

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ULTRASTRUCTURAL OBSERVATIONS ON A CARP BLOOD PARASITE OF UNCERTAIN TAXONOMIC POSITION

J.M. BUCSEK, GY. CSABA

Central Veterinary Institute, Budapest, Hungary

ABSTRACT

Authors present the ultrastructure of a protozoon living in carp fry blood. This protozoon was investigated on several occasions under the light microscope by Smirnova /1971/, Kudriashova and Naumova /1978/ and they described it as Haemogregarina cyprini. The protozoon in this paper seems to be the same, but because of the absence of certain cellular organelles and its extracellular occurrence it may not be a member of Haemogregarinae.

INTRODUCTION

Blood smears of carp fry are more frequently examined to clear the aetiology of fish death and losses of unexplained origin under intensive culture conditions.

Smirnova described a new species as Haemogregarina cyprini in 1971. Francini and Saini /1923/ reported on a Haemogregarina from the scraping of carp bowels, but their Haemogregarina carpionis turned out to be a coccidium. Csaba found a protozoon in 1976 resembling Smirnova's Haemogregarina, but he did not consider it as such, because he could observe only extracellular developmental forms in the blood samples of carp fry. Kudriashova and Naumova /1978/ reported on the pathogenic effect of Haemogregarina cyprini on carp. The protozoon in their paper seems to be the same as that described by Csaba. Molnár /1980/ refers to the presence of the same protozoon associated with carp sphaerosporosis.

Ultrastructural studies of the protozoon were performed in order to decide whether it contains organelles making its classification among Haemogregarinas possible and if it is really extracellular.

MATERIAL AND METHODS

Ultrastructural examination was performed on first-summer carps that contained 5-6 protozoa per microscope field of blood smears when studied at high magnification. Samples were taken from heart, gill, kidney, spleen and blood. Blood samples were prepared according to our method: blood was taken from the tail artery with a glass capillary and after clotting it was blown out from the capillary to the fixative. All samples were fixed for 2 hours in 5 % glutaraldehyde solution buffered with sodium cacodylate /pH 7.4/ then rinsed in the same buffer, postfixed in cold, 1 % osmium tetroxide for 1 hour, dehydrated in ascending series of ethanol, and embedded in Durcupan ACM. Thin sections were made and stained with uranyl-acetate and lead citrate and examined under a Philips 201 CS electron microscope.

RESULTS

Protozoon was found in all the samples and always extracellularly inside capillaries /Fig. 1/. It had distinctive features from host cells. Its cell surface differed from the host cell surface, its plasmalemma was of thicker type, the chromatin of the nuclei was more homogenous than that of the host and it had strikingly large nucleoli. There were relatively few mitochondria in the cytoplasm and these were of vesicular type, characteristic of unicellular organisms. They cytoplasm contained free ribosomes and rough endoplasmic reticulum. Cysternae of granular endoplasmic reticulum located concentrically around the nuclei and around the secunder cells arising inside the mother cells. Golgi apparati occurred, but no centrioles could be observed /Figs 2, 7/. Microtubules could often be seen either subjacent to the cell membrane /Fig. 3/ or inside the cell /Fig. 4/. It was common that besides the nucleus there were also some secunder cells in the cytoplasm of the mother cell and division of secunder cells could be recognised on several occasions /Fig. 5/.

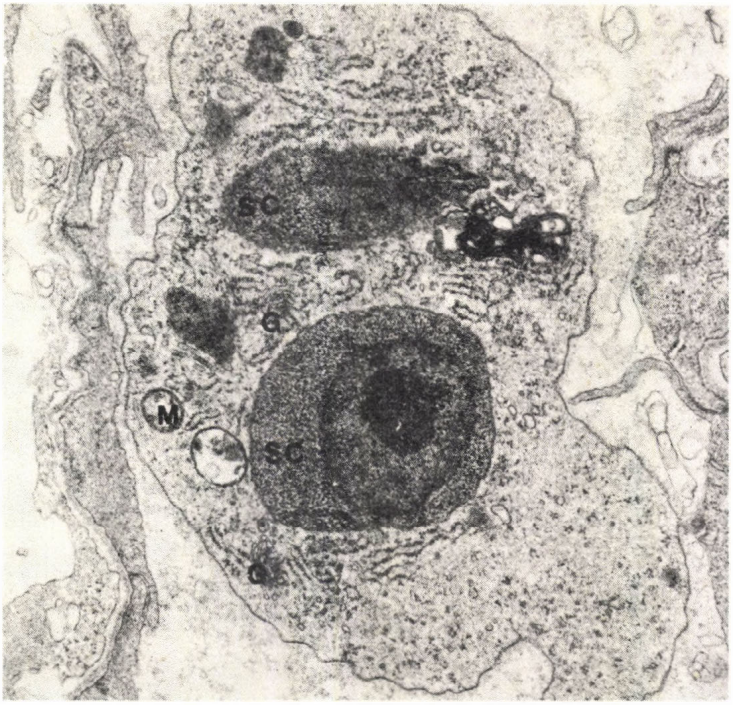

Figure 1. Inside of capillary there is a protozoon containing two secunder cells in the cytoplasm in addition to mitochondria, Golgi apparatus and granular endoplasmic reticulum /x 22,000/. MC: mother cell; SC: secunder cell; N: nucleus; M: mitochondrium; G: Golgi apparatus; Mt: microtubules; Rb: residual body; E: red blood cell

These daughter cells were separated by a single or double membrane from the cytoplasm of the mother cell. In addition to secunder cells, residual bodies /as they were called after the light microscopic examination/ could also be found. They were dark, surrounded by a membrane and contained proper internal structure formations /Fig. 6/. The protozoon never contained polar ring, conoid, micronema or rhoptries.

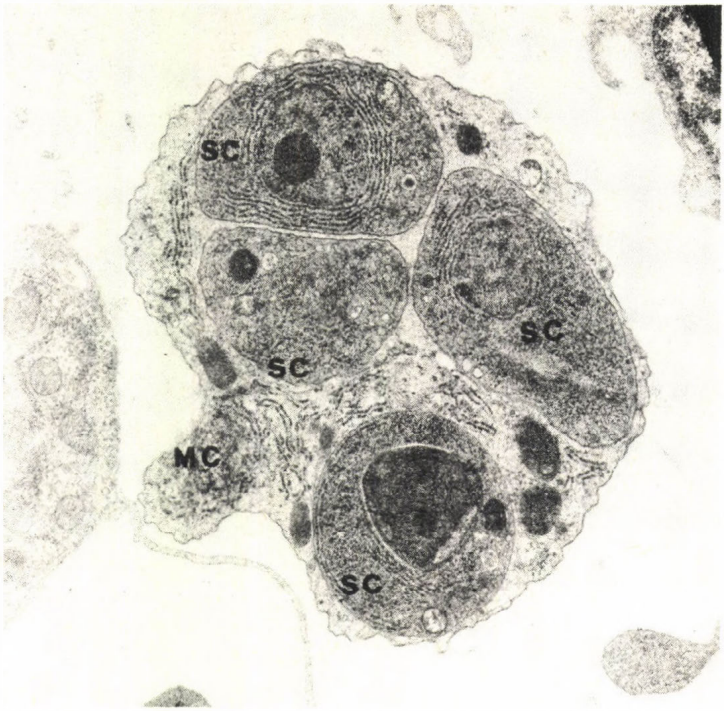

Figure 2 Mother cell containing 4 daughter cells. They are surrounded by a single membrane /x 14,000/. For details cf. Fig. 1

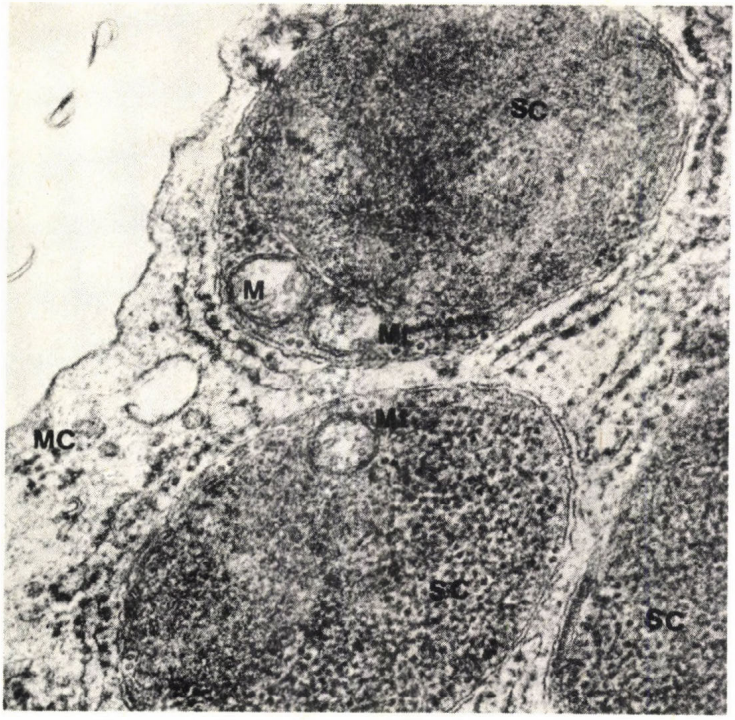

Figure 3 Parts of 3 daughter cells. Subjacent to the double limiting membrane note the cross-sections of microtubules in irregular arrangement /x 68,000/. For details cf. Fig. 1

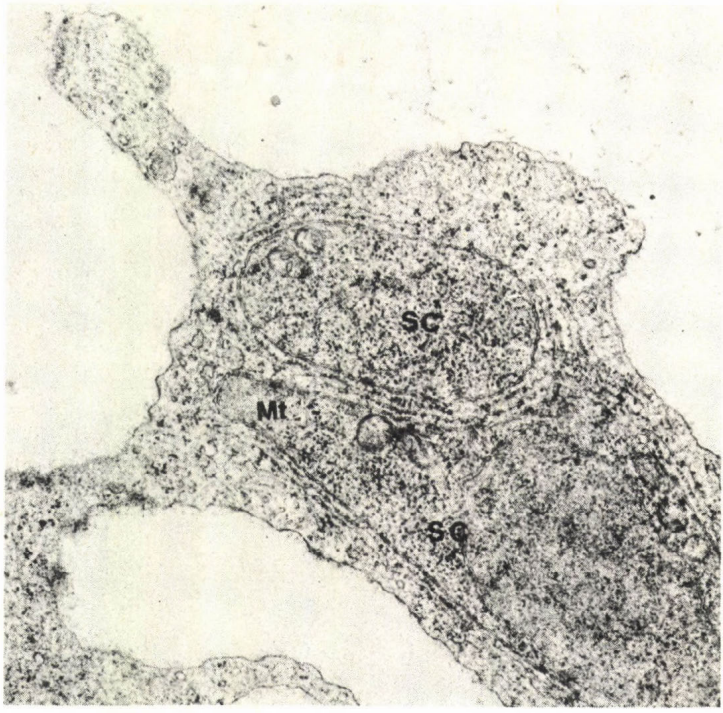

Figure 4 In the apical pole of a daughter cell cross-sections of microtubules can be seen. There is no conoid in the daughter cell /x 28,500/. For details cf. Fig. 1

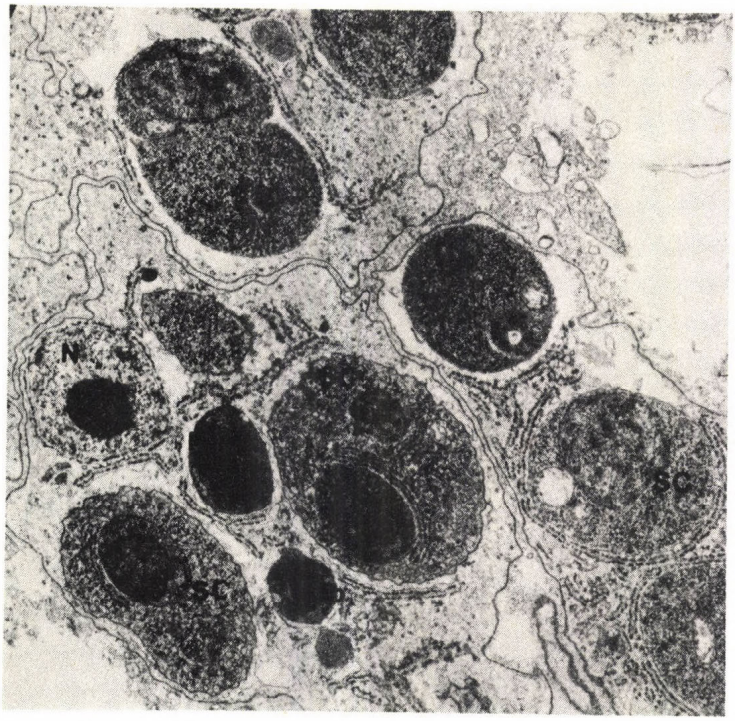

Figure 5 Secunder cells are in the final phase of division /x 44,000/. For details cf. Fig. 1

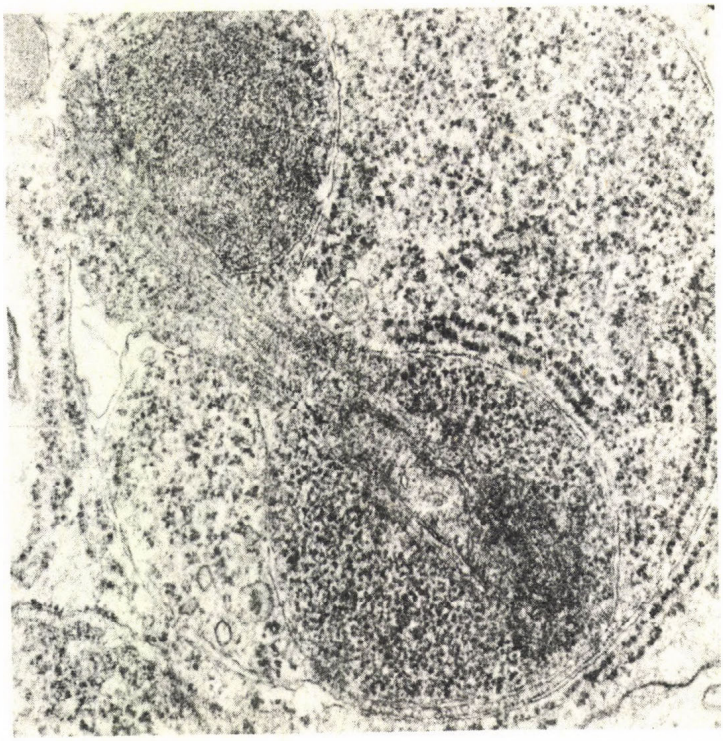

Figure 6 A group of protozoon. There are residual bodies in one of them in addition to the daughter cells and nucleus /x 14,000/

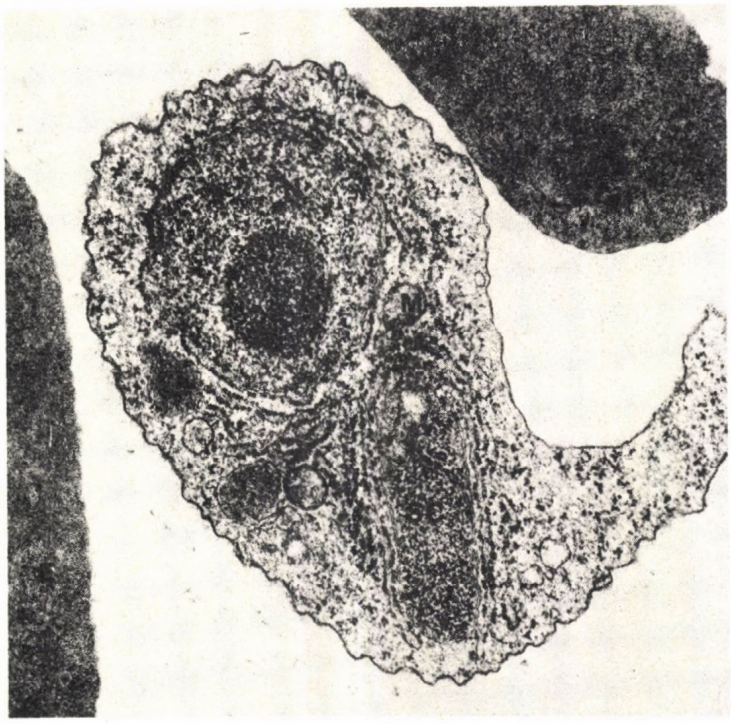

Figure 7 Between parts of two red blood cells there is a protozoon containing secunder cells next to the nucleus. Around the nucleus and the secunder cell note the granular endoplasmic reticulum in circular arrangement /x 30,000/. For details cf. Fig. 1

DISCUSSION

The fine structure of a protozoon of uncertain taxonomic site has been reported by several authors. Daniels et al. /1976/ described an intracellular protozoon living in the skin of rainbow trout. After penetrating the host cell, this protozoon produced some daughter cells within itself, that multiplied later on as a result of repeated simultaneous divisions. In their opinion, the secunder cell development may be a modified form of schizogonia. The protozoon observed by us occurred free in the blood, outside the host cells, however, the appearance of daughter cells remind us to a certain extent of the protozoon described by Daniels et al. /1976/. For the same reason we have tried to find a resemblance to the unknown protozoon living in spinal myelinated axons of Bufo arenarum /Stensaas et al., 1967/.

The early phase of development of the protozoon described in our study shows some resemblance to the phase preceding sporogony of the members of Myxosporidia reported in a study of Grasse and Lavette /1978/ on Sphaeromyxa sabrazesi.

Considering that the carp blood protozoon studied here was always found extracellularly, that it does not have polar ring, conoid, paired organelles and micropore; it cannot be a member of Haemogregarinae. Haemogregarina cyprini described by Russian authors may be the same protozoon as the one studied in our paper. Intracellular forms in the red blood cells studied under the light microscope were seldom found.

We consider it probable from the ultrastructural observations reported here, that this protozoon can be regarded as the member of Myxosporidia, but further studies on sporulation should decide this.

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OCCURRENCE, SPREAD AND CONTROL OF BOTHRIOCEPHALUS ACHEILOGNATHI IN THE CARP PONDS OF THE GERMAN DEMOCRATIC REPUBLIC

F. WEIROWSKI

Fish Health Service of the GDR, Berlin, GDR

ABSTRACT

Since the first case when Bothriocephalus acheilognathi Yamaguti 1934 /Syn. B. gowkongensis/ /Cestoda Pseudophyllidae/ was diagnosed, experts of the Fish Health Service of the GDR have been constantly analysing the epizootiology of bothriocephalosis. Tests revealed the incidence of bothriocephalosis in 220 carp farms, i.e. 13 % of the total pond area of the GDR. In the last five years, the average extent of infestation was about 14.5 % of the fish stocks affected. The intensity of infestation reached an average of 2.6 tapeworms per fish. Under our climatic conditions it has spread slowly and host-parasite relationship has levelled off resulting in relatively constant infestation rates. Carp production is endangered only if stocks are heavily infested. Bothriocephalosis has only little economic relevance if government injunctions are effectively coordinated with measures of prophylaxis, active therapy and permanent veterinary control of fish stocks.

INTRODUCTION

In the GDR Bothriocephalus acheilognathi Yamaguti, 1934 /Syn. B. gowkongensis/ Cestoda Pseudophyllidae was first diagnosed in a carp farm in 1976. Since then complex prophylactic and therapeutic measures have been taken to prevent the further spread of tapeworms and to keep economic losses in the affected fish stocks at a low level. Since the first case, when bothriocephalosis was diagnosed the Fish Health Service of the GDR has

permanently analysed the epizootiology of Bothriocephalosis. In this way the necessary measures can be coordinated centrally and limited to an expenditure which is economically justified.

MATERIAL AND METHODS

The present analysis has considered all the economically relevant carp stocks of the GDR. One- and two-year-old carp stocks were regularly subjected to routine health checks at intervals of 14 to 21 days. 6 to 10 fish were investigated in each case. Edible fish were investigated at least once a month. These investigations revealed some 3,000 cases per year from which only those test findings were considered in this analysis which showed a clear incidence of bothriocephalosis. Special parasitological tests were conducted to establish the extent and intensity of bothriocephalosis in fish farms where the infestation was obvious.

The health condition of fish was recorded through clinical--anatomical-haematological, parasitological and bacteriological diagnoses. Losses of fish caused by tapeworm infestation were also registered. The extent of infestation indicates the proportion of infested fish in a stock. The intensity was defined as the average number of tapeworms found in infested fish.

RESULTS

1. Spread. Bothriocephalus acheilognathi was introduced by the import of edible carps and grass carps. Since the first incidence of bothriocephalosis in 1976, a steady and slow spread of bothriocephalosis has been observed in various carp farms. The main channels through which bothriocephalosis spreads are the flowing waters with copepods, wild fish and to a lesser degree birds. There is a flourishing trade in young fish among carp farms. Strict government control is enforced to prevent the active spread of tapeworms through this trade. Trade in edible carps from areas infested with bothriocephalosis is also subject to strict control. Therefore, the incidence of bothriocephalosis in carp farms has so far been relatively low.

Tests revealed the incidence of bothriocephalosis in 216 traditional carp ponds, in 2 farms with intensive management, in effluent water of power stations, and in 2 cage farms.

Fish stocks in these affected rearing systems account for 9 % of the total production of carp fingerling and 12 % of the total production of carp for market. The affected ponds and farms account for 13 % of the total pond area and about 3 % of the total lake area of the GDR. Important rivers, however, such as the Neisse, the Spree and the Elbe are fed with water from these areas, so that they are potential channels for spreading bothriocephalosis.

2. Extent and intensity of infestation. The average infestation rates established have been constantly low over several years. In the last five years the average extent of infestation was about 14.5 % of the fish stocks affected. In the same period intensity of infestation reached an average of 2.6 tapeworms per fish. These values varied from year to year, the extent of infestation, for example, ranged from 8.4 to 29.1 %, while the intensity of infestation varied from 1.6 to 3.4 tapeworms per fish. The values given were established from June till August, i.e. when the incidence was at its highest. Only in farms with intensive management - where water circulation is used - the extent was as high as 45 to 80 % while for short periods the intensity of infestation in the same period reached a level of 10 to 25 tapeworms per fish.

In many ponds the tapeworm, Cestoda Khawia Sinensis HSÜ occurs together with Bothriocephalus. This makes the assessment of losses caused by bothriocephalosis more difficult.

3. Growth of fish. Fish harvests showed that in heavily infested ponds and warm water farms the individual weight of fish was somewhat lower than the weight of fish in comparable fish stocks. In heavily infested carp farms /more than 3 tapeworms per fish, extent more than 20 %/ the average individual weight of edible carp was 10 % lower compared to the weight of carps not affected. Low infestation rates, below 2 tapeworms had no tangible pathogenic effect on either young fish or edible carps.

4. Feeding costs. In heavily infested stocks feeding costs increased by 5 % compared with non-infested stock.

5. State of health. In ponds with low infestation rates a clear pathogenic effect could not be detected. Heavy infestation in ponds and warm water farms, however, leads to a decrease in the serum protein content. At infestation rates of 40 % and 3 to 5 tapeworms per fish the haemoglobin content in the blood of one-year-old carps decreased by 7.3 %. Even the number of erythrocytes decreased. Heavy infestation causes chronic enteritis, intestinal blockage, inflammation and perforation. This results in a higher susceptibility of bothriocephalosis-infested fish to other infectious diseases.

6. Losses. The analyses showed that losses of fish caused directly by bothriocephalosis are very low. In most cases a deterioration in the general state of health is responsible for losses through secondary diseases. It is estimated that on the average, carp production suffers losses through bothriocephalosis in 1-1.5 % annually. In warm water farms with intensive management, losses are somewhat higher.

7. Control. Prophylactic measures include, above all, the control of trade in bothriocephalosis-infested stocks, the disinfection of ponds after fish harvest and the use of parasite-free breeding stock. By isolating infested fish stocks the active spread of bothriocephalosis through farming can be stopped. For this purpose quarantined zones are demarcated. In the case of infested fish stocks therapeutic measures must be taken without delay. The fish are fed on pellets impregnated with anthelmintic "zestocarp" /active agent niclosamide/. The greatest effect can be achieved in summer /June - September/. In this way the intensity and the extent of infestation can be decreased to 0-10 % of the previous level. In warm water fish farms infestation could be reduced by 80-100 %. It is possible to kill simultaneously intermediate hosts - copepods - with 0.25 ppm Trichlorphon Masoten, Dipterex/. In warm water fish farms the therapeutic effect was higher but only by permanent repeating of the treatment. This, however, is not the most economic.

Ponds and farms are disinfected after fish harvest, 1,000 kg hydrochloride per hectare is sufficient. Where it is possible, ponds are drained so that the moisture content amounts to about

10 % of the surface layer. Freezing the ponds in winter has also proved effective. After fish harvest vehicles and implements are disinfected with meleusol, a 4-per-cent solution of cresomelate.

Edible carps infested with bothriocephalosis have to be fed on pellets for at least 28 days before harvest to avoid complaints by the consumers. Trade in edible carps from ponds with clear evidence of bothriocephalosis is allowed only for processing purposes, in cities with sewerage and in rural areas where tapeworms have already spread over wide areas. These injunctions are complied with by farms when edible carps are put on the market.

DISCUSSION

Under the climatic conditions prevailing in the GDR bothriocephalosis has spread only slowly. Strict government control measures played a considerable part in keeping the spread of bothriocephalosis in the carp stocks of the GDR at a low level. Under natural conditions the host-parasite relationship has levelled off from which relatively constant infestation rates resulted, with an extent of 15 per cent and an intensity ranging from 2 to 3 tapeworms per fish. High temperature, the use of water circulation and a high number of fish per pond meant a greater danger to the health of fish in warm water farms.

In general, carp production is endangered only if stocks are heavily infested, i.e. if the extent is 20 % and the intensity is 3 tapeworms per fish. If the balance is upset, i.e. if constant infestation rates are exceeded, the health condition of the fish is impaired and enteritis as well as a greater susceptibility to infectious diseases develop.

The means and methods of control currently available have been successful so higher losses could be avoided and the spread of tapeworms to all the farms could be prevented. Complete eradication of tapeworms in infested areas could not be achieved, because of the various channels in which eggs and proceroids are spread by copepodes in rivers. Control measures such as disinfection of ponds and draining systems, monitoring young fish, and the control of trade in edible fish can slow down this process. They are regarded to be necessary, and will be continued because the economic expenditure on the control of bothriocepha-

losis means an additional burden for the farms. Feeding fish on anthelmintic pellets has been very effective.

CONCLUSIONS

After 5 years' study of bothriocephalosis we have come to the conclusion that bothriocephalosis is only of little economic relevance if government injunctions are effectively coordinated with prophylactic measures, active therapy and permanent veterinary control of fish stocks.

If, however, control if neglected, bothriocephalosis will spread rapidly and seriously affect stocks, especially in warm water fish farms with intensive management.

The findings obtained are currently summed up in the form of directions for legislation governing epizootics.

This ensures that control, diagnosis, prophylaxis and therapeutic measures be standardised and binding upon all farms.

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NEW HELMINTHOSES OF THE CARP IN THE CARPATHIAN REGION OF CZECHOSLOVAKIA

R. ŽITŇAN

Helminthological Institute of the Slovak Academy of Sciences, Košice, Czechoslovakia

ABSTRACT

The objective of the work was to study the distribution /1976-1977/ and to elucidate the outset and formation of first invasions /1978-1979/ induced by new helminthosis agents Gyrodactylus schulmani /Monogenea/ and Bothriocephalus gowkongensis /Cestoda/ in the carp fry under the ecological conditions of the Carpathian region of Czechoslovakia.

It has been found that G. schulmani is distributed over 26 localities and its invasion cycle is dynamic. The mean values of extensity and intensity of invasion increase gradually from the first invasions of the carp fry, reach their maximum in the warmest summer season, and then decrease gradually down to their minimum in the autumn. In the winter season only sporadic parasite findings were recorded.

It has also been detected that B. gowkongensis is distributed over 10 localities and its invasion cycle is of the dynamic character as well. The mean values of extensity and intensity of invasion increase gradually from the first invasions of the carp fry until the first half of August when they reach their maxima.

Later the invasion of fish decreases gradually until the end of the vegetation period. Following the transfer of fish for wintering, extensity and intensity of invasion do not decrease markedly, and during the winter period no destrobilization of B. gowkongensis occur.

INTRODUCTION

The basis of freshwater fish production in Czechoslovakia is the carp, which represents approximately 90 % of the annual fish production. Under the conditions of intensive management, one of the basic factors conditioning successful carp rearing is the good health condition of the reared fish. Even at present, it is obvious that the intensification in carp rearing /increased stocking density, feeding, increased application of organic and mineral fertilizers, etc./ brings about a whole complex of new problems also in the Carpathian region of Czechoslovakia, among which the occurrence of invasive diseases, namely some helminthoses may also be ranked.

Prior to our studies, the knowledge on the carp helminths in the Carpathian region of Czechoslovakia originated mostly from the study of running water, where in the majority of instances carp was not considered among the economically most significant fish species. In such waters 27 helminth species were detected in the carp /Michalovič 1954, Kašťák 1955, 1957, Vojtek 1959, Žitňan 1960, 1964, 1965a, 1965b, 1966a, 1966b, 1966c, 1967, 1968a, 1968b, 1968c, 1969, 1970, 1974a, 1974b, 1977, 1979a, Ergens et al. 1975/.

Systematic studies on helminths and helminthoses of carp in ponds and water reservoirs were commenced as late as in 1976. These observations have revealed /Žitňan 1978, 1979b, 1979c/ that among 18 detected helminth species also 2 new species /Gyrodactylus schulmani Ling Mo-en, 1962 and Bothriocephalus gowkongensis Yeh, 1955/ are present, which have been widely distributed recently and may hamper considerably further intensification of the carp rearing.

MATERIAL AND METHODS

In the study on the species structure of carp helminths, in 1976 and 1977, from different types of waters in the Carpathian region of Czechoslovakia a complete helminthologic autopsy was employed in examination of 1852 carps caught in 42 different localities /20 ponds, 15 basin reservoirs, 6 dams/. Besides the mentioned fish, other 1007 carps from 32 localities /at least 20 specimens at a time/ were examined in order to define the distribution of B. gowkongensis more precisely.

In the study on the seasonal dynamics of the invasion cycle of the economically most significant helminths of the carp fry, 860 carps were examined by means of complete helminthological autopsy in all the seasons of 1978 and 1979. The fish were from three ponds /Southern, Northern and Wintering/ of the fish farm at Perin in the Moldavian Lowlands, situated 196.50 m above sea level. In the vegetation period, the fry were examined in 10-day intervals / in 1979 even in shorter intervals/ and during the wintering in one-month intervals, 20 specimens at a time. The autopsies were performed immediately after fishing out and only live, undamaged fish, maintained in a sufficient amount of water for a short time and caught in the pond under study were examined.

RESULTS

On account of numerous data obtained from the study this paper presents a brief evaluation only of the results concerning distribution and the seasonal dynamics of the invasion cycle of G. schulmani and B. gowkongensis.

Distribution and seasonal dynamics of the invasion cycle of Gyrodactylus schulmani

Distribution of G. schulmani in the studied types of waters is illustrated in Figure 1. In the years of 1976 and 1977, this species was found in as many as 26 localities, namely in 14 ponds /within 7 fish farms/, 9 basin reservoirs and 3 dams.

During the study on the seasonal dynamics of the invasion cycle, the first finding of G. schulmani was recorded from Southern Pond in 1978 on the 18th-19th day after hatching of the fry, with body length of 25 mm. Figure 2 presents the seasonal dynamics of the invasion cycle of G. schulmani in Southern Pond during the vegetation period of 1978. The invasion cycle curve rapidly increases from the appearance of the first helminths after hatching of the carp fry till the beginning of August when it culminates at the maximum extensity values /100 %/ and the average invasion intensity /33.8 specimens/. Then the invasion gradually decreases reaching the minimum values /extensity 5 %, intensity 1 specimen/ by the end of October, closely before the autumn fishing out.

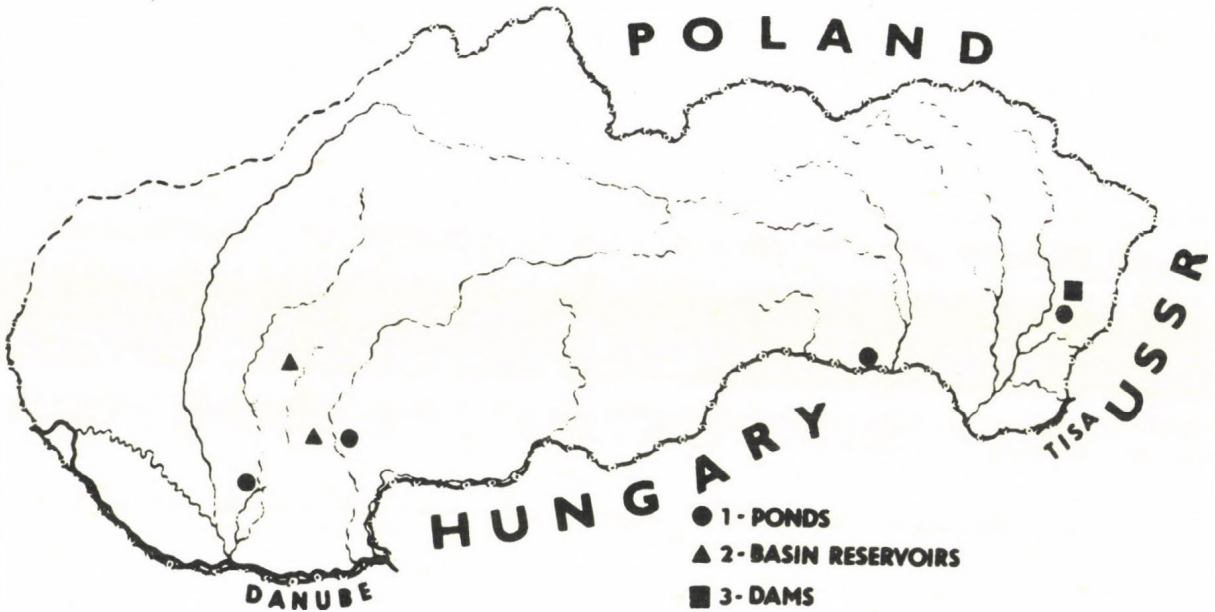

Figure 1 Distribution of Gyrodactylus schulmani in the studied types of waters in the Carpathian region of Czechoslovakia

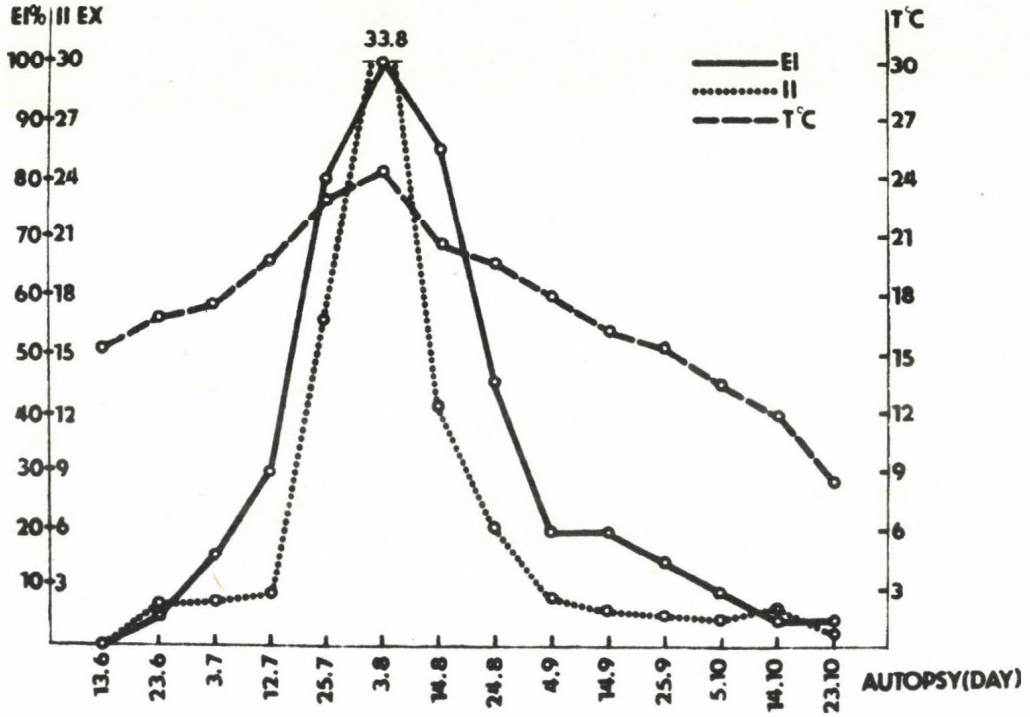

Figure 2 The seasonal dynamics of the invasion cycle of Gyrodactylus schulmani in the carp fry from the southern pond during the vegetation period of 1978

The seasonal dynamics of the invasion cycle of G. schulmani in Northern Pond in the vegetation period of 1979 is illustrated in Figure 3. Similarly to the previous year, the invasion extensity and intensity rapidly increase from the first invasions in June, reaching the maximum values in July /extensity 100 %, average intensity 29.1 specimens/.

After the summer maximum the gradual decrease in both the invasion indices occurs, coming down to the zero values at the beginning of November.

In the course of the winter season of 1978-1979 /January - April/, there was no evidence of G. schulmani at all in the group of 80 examined fishes from wintering pond. Results of 140 complete helminthological autopsies, carried out from November, 1978 till the end of April, 1979, have also confirmed that invasion of the wintering carps with this parasite species is very low /extensity 0.70 %, intensity 2 specimens/, only a single case of its occurrence was recorded.

Distribution and the seasonal dynamics of the invasion cycle of Bothriocephalus gowkongensis

Distribution of B. gowkongensis in the studied types of waters is given in Figure 4. In 1976 and 1977, this species was detected in 10 localities, namely in 7 ponds /within 3 fish farms/, 2 basin reservoirs and 1 dam.

In the study on the seasonal dynamics of the invasion cycle, the first finding of B. gowkongensis was recorded from southern pond in 1978 on the 28th-29th day after hatching of the fry, at its body length of 38-42 mm. A gradual increase could be observed in the extensity and intensity of invasion of the fry from the first invasions till the first half of August /Fig. 5/, when the maximum values were reached /extensity 55 %, average intensity 4.0 specimens/.

Following this maximum the invasion slightly, but gradually decreased and by the end of October, prior to the autumn fishing out, it fell down to the minimum values /extensity 40 %, average intensity 2.6 specimens/.

During the winter season of 1978 and 1979 /Figure 6/ the course of the invasion extensity and intensity was as follows: from transfer of fish into Wintering Pond in November, 1978

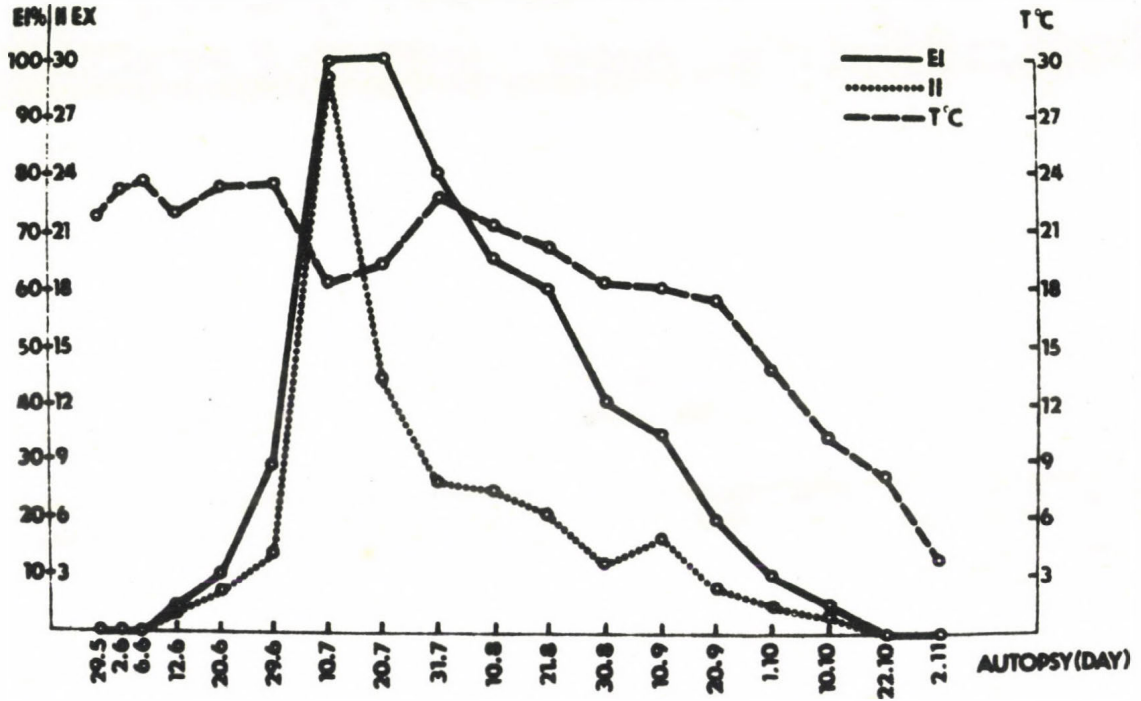

Figure 3 The seasonal dynamics of the invasion cycle of Gyrodactylus schulmani in the carp fry from the northern ponds during the vegetation period of 1979

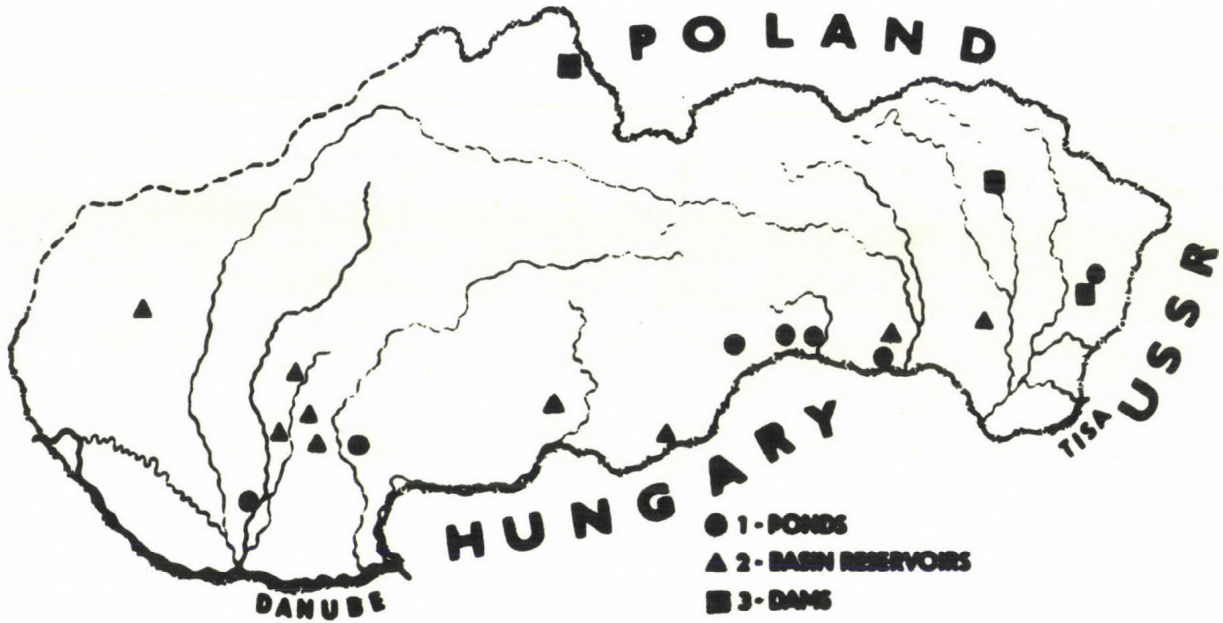

Figure 4 Distribution of Bothriocephalus gowkongensis in the studied types of waters in the Carpathian region of Czechoslovakia

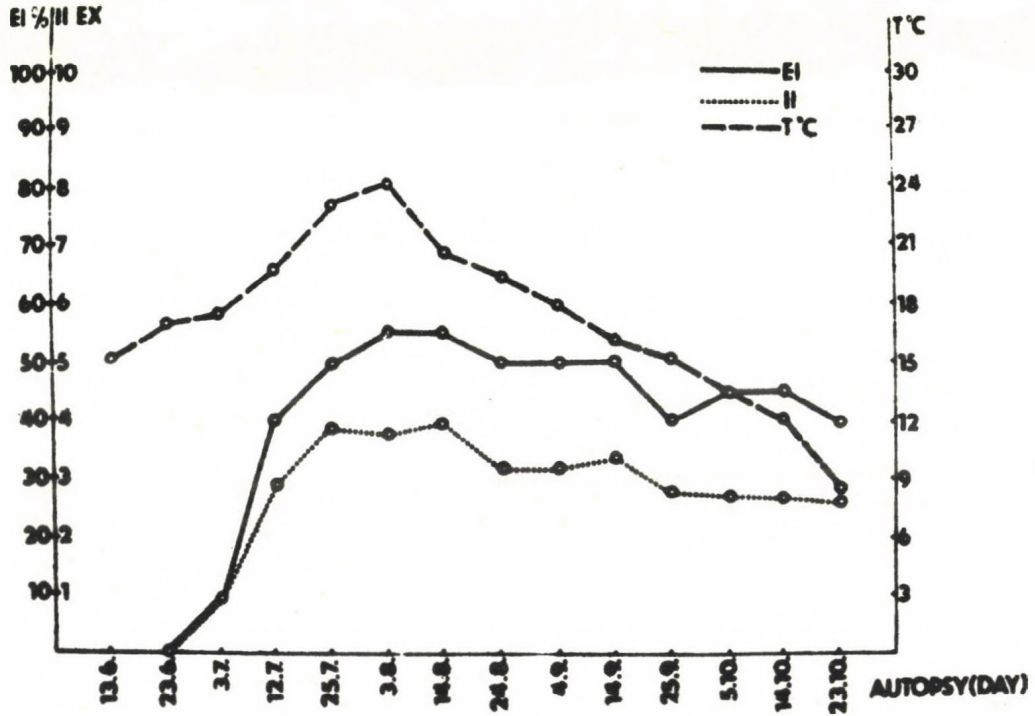

Figure 5 The seasonal dynamics of the invasion cycle of Bothriocephalus gowkongensis in the carp fry from the southern pond during the vegetation period of 1978

the extensity of invasion remained approximately at the same level in the course of the entire winter season till April, 1979. Also an average intensity of invasion, though indicating the decreasing tendency /4.0-3.1/, did not decrease significantly. /Fig. 6/.

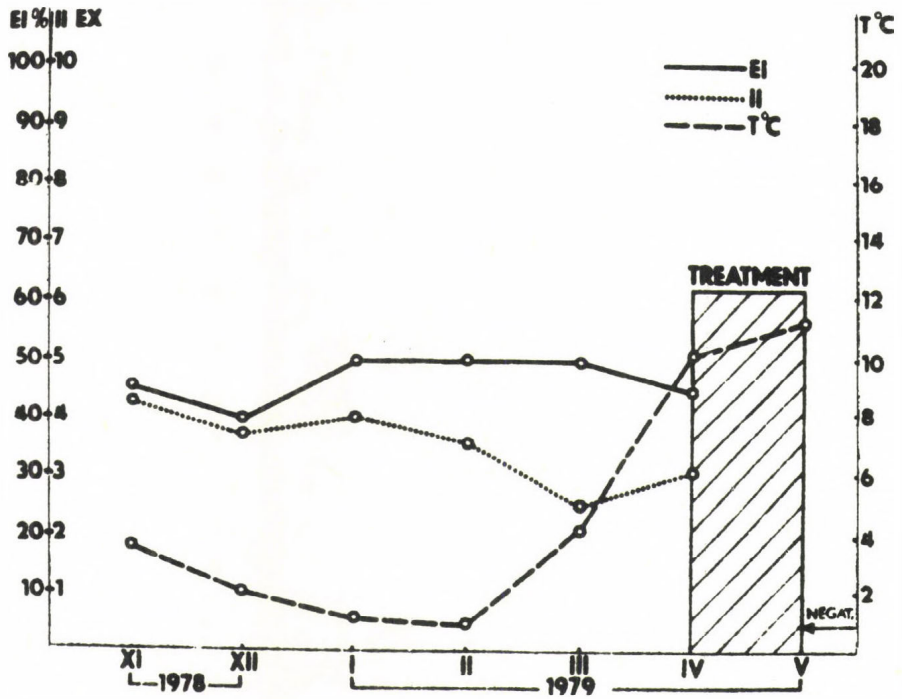

Figure 6 The seasonal dynamics of the invasion cycle of Bothriocephalus gowkongensis in the course of the first wintering of carps in the Wintering Pond in 1978-1979

Autopsies performed during May revealed negative results as the consequence of the successful dehelminthization of fish with the drug, Taenifugin carp SPOFA, applied in Wintering Pond from the end of April till the beginning of May, 1979.

DISCUSSION

G. schulmani, until only recently the unknown representative of the Gyrodactylus genus in the Carpathian region of Czechoslovakia was first described by Ling Mo-en /1962/ from gill filaments of Carassius auratus, caught in the river Liao-Ho in China. Its first finding in our country was recorded in 1964 on the fins of Carassius carassius from the Tisza, and since we had no knowledge of the work by Ling Mo-en, the collected specimens were described as Gyrodactylus sp. 2 /Žitňan 1966a, 1968 d/. This epizootiologically significant parasite has spread considerably in recent years. It is assumed that its distribution in waters of the Carpathian region of Czechoslovakia is closely connected with the purposeless introduction /for acclimatization of herbivorous fish/ or the natural expansive distribution of a new form of Carassius auratus in the river basin of the Danube /Holčík and Žitňan 1978/. G. schulmani parasitizes on gill filaments, gill arches, skin and fins of the carp /Cyprinus carpio L./ and in its phylogenetically closely related fish species /Carassius carassius, Carassius auratus/, and penetrating from waters - feeding rearing ponds - becomes the vector of the helminth.

The seasonal dynamics of the invasion cycle of G. schulmani has a one-peak curve of dynamic character /the mean values of the invasion extensity and intensity are on the increase from the first findings of the helminth, reaching the maximum in the warmest summer season, and then decreasing gradually to the minimum in autumn/. Since the first finding of G. schulmani was reported on the 18th-19th day /1978/ or on the 22nd-23rd day /1979/ after hatching of the fry, it is considered to be a parasite with an early outset of invasion. Under optimal climatic conditions, the extensity and intensity of invasion may reach the maximum values as early as the beginning of July, when carp fry are relatively small and may be seriously damaged.

Our findings also point out the consequence of this new gyrodactylosis, which is often accompanied with dactylogyrosis, whose agent, Dactylogyrus vastator, is an epizootiologically significant gill parasite of the carp fry. Dactylogyrus vastator, like G. schulmani, reaches the maximum values of the inva-

sion extensity and intensity in the warmest summer season in the fry of approximately the same age and size, and may contribute significantly to the losses, especially to the density of cultures under the conditions of carp rearing intensification.

Studies on the formation of the first helminth invasions, on the changes in invasion during the vegetation period and wintering of the carp fry along with the basic epizootiological data on G. schulmani may serve as a basis for the planning and realization of the prophylactic dehelminthization of the fry in the season preceding the maximum of the invasion cycle.

Nowadays, in the literature there are so many data on distribution, morphology, biology, intermediate hosts, hosts, diagnostics, therapy, epizootiology, ecology and the seasonal dynamics of Bothriocephalus gowkongensis that they would be impossible to cite them.

It is necessary to mention, however, that under the ecological conditions of central Europe, the morphology of the parasite was studied by Molnár and Murai /1973/ and Pár and Párova /1976/, who stated that B. gowkongensis is probably the synonym of Bothriocephalus acheilognathi Yamaguti, 1934. In our view, this problem could be explicitly solved only on the basis of the comparative study of the material collected from the entire distribution area of the parasite.

The recorded seasonal dynamics of the invasion cycle of B. gowkongensis in the vegetation period of 1978 had the same course as under the similar ecological conditions of other countries. Thus, e.g. Musselius /1962, 1967/ reported from her study in the central region of the USSR that the invasion extensity and intensity of B. gowkongensis in the carp fry reached the maximum value in the midsummer /in July/. Later, when the fry convert from plankton to benthic feed /zoobenthos/, the invasion extensity and intensity are on the decrease until the end of the vegetation period. A relatively serious invasion /extensity 98.1 %, average intensity 14.6 specimens/ was detec ted in 30-day-old fry of 70 mm average body length.

In the Kiev region of the USSR, Shcherban /1965/ reported on a similar increase and decrease in invasion of the carp fry with the tapeworm B. gowkongensis during the vegetation period.

In the Moscow and the Kursk regions of the USSR, Muzykovsky /1968/ found the maximum invasion of the carp fry in August and he also observed a gradual decrease in the invasion of fish until the autumn fishing out. As to the invasion of the carp fry in the course of the winter season, no significant decrease was likewise found in the invasion extensity and intensity was recorded. He found similarly, that from November till April the extensity and intensity of B. gowkongensis invasion remained at the same level, and no destrobilization of the tapeworm occurred.

SUMMARY

In the study on distribution of new carp helminthoses in different types of waters of the Carpathian region of Czechoslovakia /ponds, basins, reservoirs, dams/, in 1976-1977, 1852 carps from 42 localities were examined by complete helminthological autopsy and 1007 carps from 32 localities were examined by organ helminthological autopsy. In the study on seasonal dynamics of the invasion cycle of Gyrodactylus schulmani /Monogenea/ and Bothriocephalus gowkongensis /Cestoda/ in the carp fry, in the course of all the seasons of 1978-1979, 860 fishes from 3 ponds of the carp rearing fish farms at Perin /the Moldavian Lowlands/ were examined by complete helminthological autopsy. The findings may be summarized as follows:

1/ The occurrence of G. schulmani was detected in 26 localities of the studied types of waters. Its distribution in the recent years is connected with the purposeless introduction or the expansive distribution of a new form of Carassius auratus in the waters of the Carpathian region of Czechoslovakia. The mentioned species is presently ranked among the epizootiologically significant helminths of the carp fry with an early outset of invasion. It invades the fry as early as on the 18th-19th day after hatching at a body length of 25 mm. The seasonal dynamics of the invasion cycle of G. schulmani is of the dynamic character and it reaches the maximum values of the invasion extensity and intensity in the warmest summer season /July, beginning of August/. A consequence of these invasions is that they are accompanied by dactylogyrosis, whose agent /Dactylogyrus vastator/ reaches the maximum values of both the invasion cycle

indices in this period as well. Following the summer maximum, the gradual decrease in the invasion extensity and intensity comes about with the minimum values at the end of the vegetation period. In the winter season only sporadic findings of the helminth were recorded.

2/ Distribution of B. gowkongensis was reported from 10 localities of the studied types of waters, and this species also belongs to the epizootiologically significant new helminth of carp in the Carpathian region of Czechoslovakia.

Studies on the seasonal dynamics of the invasion cycle of B. gowkongensis revealed the first findings of the tapeworms on the 28th-29th day after hatching of the carp fry at a length of 38-42 mm. Increase in the invasion extensity and intensity was gradual from the first invasions till the beginning of August, and after this maximum, the invasion slightly but gradually decreased till the end of the vegetation period /till the autumn fishing out/. Since the transfer of fish into Wintering Pond for wintering /in November/, the invasion extensity retained the same level during the entire winter season. The average intensity, showed a slightly decreasing tendency. No destrobilization of B. gowkongensis was observed in the course of the winter months.

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INVESTIGATION ON INVASIVE DISEASES OF THE HERBIVOROUS FISH FRY AND THEIR TREATMENT

Z. LUCKÝ

Department of Poultry, Fish, Bee and Wildlife Diseases,
Veterinary School, Brno, ČSSR

ABSTRACT

The present report evaluates the results of a parasitological investigation of the fry of silver carp /Hypophthalmichthys molitrix/, bighead carp /Aristichthys nobilis/ and grass carp /Ctenopharyngodon idella/.

In silver carp fry 15 pathogens of fish invasive diseases were found, among them Myxobolus pavlovskii, transferred into fishponds with imported fish. The other parasites were transferred by inland fish. In bighead carp fry 6 pathogens of parasitoses were registered, Myxobolus pavlovskii and Dactylogyrus nobilis transferred from imported fish, the other species belong to the local parasitofauna. In grass carp fry 8 pathogens were diagnosed but only Dactylogyrus lamellatus belongs to specific parasites of this fish species, the other ones are common parasites of fish in the investigated area. The present report evaluates all the discovered pathogens from the viewpoint of their seasonal dynamics and pathogenicity. From antiparasitic baths tested, the long-term bath of copper sulphate at a dose of 1.5-2 mg/l water for 48 hrs proved to be the best against all the pathogens of registered protozoan diseases and a dipping bath of sodium chloride at a dose of 50 g/l water for 3 min against the pathogens of Dactylogyroses. The report also evaluates technological procedures and hygienic measures with regard to prevention of invasive diseases.

INTRODUCTION

The concept of the development of freshwater fish production in the CSSR till 1990, is based on a major increase of herbivorous fish production. In 1975 and 1980, 13 and 30 tons of herbivorous fish of market size were caught and the plans for 1985 and 1990 are 200 and 500 tons, respectively.

To reach the planned high production, a specialized breeding centre was established in Pohořelice as a branch fish-farm of the State Fishery.

During veterinary measures on the above fish-farm, frequent parasitic invasions were registered in herbivorous fish fry as early as in the first year of their acclimatization resulting in slow growth and death of the fry.

At that time there was scanty and incomplete information on invasive diseases of herbivorous fish in our fishponds. For this reason, the investigation of pathogens of invasive diseases and their prevention in herbivorous fish fry was included in the research plan of our institute.

MATERIAL AND METHODS

The investigation was carried out in the Centre of State Fishery in Pohořelice during 1976-1980. This Centre is specialized in artificial spawning of all the herbivorous fish species and their breeding in the first vegetation period. In August and September, the fry is distributed in other fish-farms of the State Fishery and some other organizations.

Herbivorous fish fry for pathological investigation were caught in the months from June to September. Samples of fish were obtained during their hibernation and after it in isolated cases.

In the course of several years' investigation, 31 samples of herbivorous fish fry were examined, each of them including 10-30 heads. Heads of 386 silver carp, 45 bighead carp and 147 grass carp were examined.

The fish were examined by methodical procedures based on Skrjabin's helminthological and Dogel's parasitological dissections of fish. Parasites were determined according to Bychovskij et al. 1962, Musselius 1967 and Ergens and Lom 1970.

During the investigation of invasive diseases prevention and treatment, long-term, short-term and dipping baths were carried out in media with different concentrations and expositions.

The water temperature of a bath was 15-20°C and 1 litre water per 1 fish of approximate size of 100 mm. Fish tanks were abundantly aerated. Antiparasitic baths were carried out in malachite green of technical grade, zinc malachite green, malachite green oxalate, sodium chloride, copper sulphate.

Investigation and evaluation of some other preventive measures involving technological and hygienic procedures, disinfection, and some ecological factors of fish habitation.

RESULTS

Parasites of silver carp fry

Most samples of this fish species originated from local artificial spawners, occasionally from those imported from Hungary. The fry was transferred into prepared small ponds whose overflow drainage and fish collecting pit were partially disinfected.

Cryptobia branchialis - was diagnosed in 24 examined samples, 10-times in different extensity and intensity on the gill filaments. The strongest infection was found in several week-old fry in summer /August/. The parasites were also discovered in fish after hibernation. In summer, the diseased fish collected at inflows, refused feed and were slow in their growth.

Myxobolus pavlovskii - created small cysts on the gill epithelium. In the investigated area, this parasite occurs sporadically, and only weak invasions were observed during the first vegetation period of the fry. The invasion became more severe in the second year of age.

Ichthyophthirius multifiliis - occurred sporadically and very irregularly on skin and gills of some of the investigated samples. The highest extensity was found in the fry after hibernation at a very low intensity.

Apiosoma piscicola - was frequently diagnosed in the course of the vegetation period, the most severe invasions appeared after hibernation.

Trichodina nigra and Trichodina reticulata - these parasites were found in investigated fish only in isolated cases. In our

investigation the intensity and extensity of invasions increased strikingly only after the hibernation of the fry.

Trichodinella epizootica - was discovered in the fry particularly in the second half of the vegetation period.

Dactylogyrus sp. - larvae of monogenetic flukes appeared on the skin in isolated cases. They are supposed to be species from some other species of fish which had no possibility for further development on the fry.

Gyrodactylus sp. - larvae of this genus of monogenetic flukes parasited on the skin of the fry only sporadically. According to the size of middle hooks, the flukes were closely related to species G. elegans and G. latus, and as to the size of chitin parts of the haptor it was not species G. hypophthalmichthydis.

Diplozoon homoin - flukes multiplied on the fry intensively under breeding conditions and highest intensity was recorded in summer. Flukes were discovered also after hibernation.

Diplostomum spathaceum, D. baeri, D. indistinctum - metacercariae of this genus are the most important parasites of this fish species. Metacercariae penetrate the lens as early as in the first days of life of the fry and their extensity and intensity increases parallel with the age of the fish. Extensity usually reaches 100 % and intensity several tens of metacercariae by the end of summer.

Piscicola geometra - parasites are very rare in the fry and only after hibernation.

Argulus japonicus - are found very rarely in the fry of the investigated area.

Parasites of bighead carp fry

The fry of this fish species is not yet bred in monocultures, as the culturing of this fry has been taken up only recently. The obtained results serve only for orientation.

Myxobolus pavlovskii - small cysts of this species were found in 80 % of the fry bred as stocking fry. Their intensity was high and there were even 50-70 cysts in the gill epithelium.

Trichodina nigra - parasitized on gills and skin in isolated cases, the most massive invasions appeared after hibernation.

Trichodinella epizootica - was recorded also sporadically.

Dactylogyrus nobilis - monogenetic flukes appeared on the gills of the fry only after hibernation in spring and their number was not high.

Diplostomum spathaceum - metacercariae were often found in the lens of fish, however, their intensity was very low, and contained only few specimens /5/.

Parasites of the grass carp fry

We determined in 4 samples of the fry of this fish species as follows:

Chilodonella cyprini - appeared at low intensity in the fry early after hibernation. It was found only in isolated cases.

Ichthyophthirius multifiliis - was diagnosed only after hibernation.

Apiosoma piscicola, Trichodina nigra, T. domerguei F. magna - parasitized in the fry very often, mostly in slight invasions.

Dactylogyrus lamellatus - occurred in the fry in isolated cases and only in the autumn and after the hibernation of the fry. The extensity was 100 %, the intensity up to 160 flukes.

Diplostomum spathaceum - infestation of the fry with metacercariae was rather high by the end of the vegetation period of the fry. Intensity may include several tens of parasites. The effected lens are usually dimmed and opaque.

Antiparasitic baths

Technical grade malachite green /imported from the USSR/ was not toxic to silver carp in a concentration of 0.025 mg/l and to grass carp at 0.05 mg/l in a 48-hour-long bath.

The above concentrations fully killed parasites of Trichodina and Trichodinella genus, but were ineffective against Cryptobia branchialis and Dactylogyrus lamellatus.

Malachite green oxalate in a long-term bath /48 hrs/ was not toxic to both species of the fish in a concentration of 0.3 mg/l. The therapeutic effect was similar to that of technical grade malachite green.

Zinc malachite green in a long-term bath /48 hrs/ was not toxic to both species of fish in a concentration of 0.3 mg/l. The therapeutic effect was similar to that of technical malachite green.

Brilliant green in a long-term bath /48 hours/ was not toxic to silver carp in a concentration of 0.1 mg/l. The used baths were ineffective.

Sodium chloride was not toxic to both the species of fish in concentrations of 50 g/l /3 min/ and 30 g/l /15 min/, respectively. The fry tolerated the concentration of 0.7 g/l in a prolonged bath. A bath in a 5 % solution for 3 min, was toxic to monogenetic flukes of genus Dactylogyrus and protozoa genus Trichodina.

Copper sulphate was not toxic to silver carp in a concentration of 1.5 mg/l in 1-week-long bath, to grass carp in a concentration of 2 mg/l. The bath was effective against Cryptobia branchialis and parasites of genus Trichodina.

DISCUSSION

It follows from the investigations that the method of artificial spawning of fish markedly prevent the transfer of parasites from mothers to their progeny. The early stages of the fry are affected only after their transfer with various species of parasites which are either transferred or persist in the fish pond.

The investigation discovered that most pathogens of invasive diseases come from the local fauna of cultured or trash fishes. Myxobolus pavlovskii, Dactylogyrus lamellatus and Dactylogyrus nobilis belong to the specific species of parasites imported to the area with herbivorous fish.

The most important invasive disease in the investigated herbivorous fish fry is diplostomosis caused by various species of metacercariae of genus Diplostomum. While the local carps are rather immune to eye metacercariae in the first year of life, herbivorous fry is very susceptible and is killed by parasitosis at an early age. The affected fish lose their ability of orientation and easily become preys of carnivorous birds. Intensive food intake is also considerably limited in an affected, nearly blind fish.

Some species of worms /Pisciccla/ and arthropoda /Argulas/ parasitized on the fry only by chance.

The best therapeutic effect was reached by a long-term bath in the solution of copper sulphate at a dose of 2 mg/l or 1.5 mg/l according to the species of fish. A 48-hour-long bath may be recommended against gill cryptobiosis as well as against all species of protozoa of genus Trichodina and Trichodinella. Silver carp is more susceptible.

The baths in the solutions of malachite greens are toxic to parasites of genus Trichodina and Trichodinella, while parasites of genus Cryptobia are not killed even after a 48-hour-long bath and their movements are nimble even after 60 hrs.

The bath in the solution of brilliant green, recommended in the literature against protozoa and some other parasites /Bauer et al. 1969/ in a concentration of 0.1-0.2 mg/l of 100 % preparation, in our experiments gave different results. Grass carp fry tolerated concentration of only 0.1 mg/l, silver carp tolerated concentration of only 0.05 mg/l for longer time. After a 48-hour-long bath all species of protozoa and monogenetic flukes were still alive.

Dipping bath in sodium chloride at a dose of 50 g/l, which killed flukes at the exposition of 3 min showed a good therapeutic effect against monogenetic flukes of genus Dactylogyrus.

Prior to filling fishponds, the wet places were layered with slaked lime to kill water slugs. Thus, the appearance of eye metacercariae was more markedly limited to isolated cases. Slugs came into tanks again with water filling the fishpond. The water continued to be further infested with cercariae of genus Diplostomum from the natural localities with flowing water, the inflow of which could not be stopped fully for operation reasons.

On the basis of our investigation we can recommend the following preventive and therapeutic measures:

1. To propagate herbivorous fish by means of artificial spawn which prevents a direct transfer of all the pathogens of invasive diseases.

2. To transfer fry in early stages into hygienic environment free from water slugs.

3. To prevent the penetration of water slugs into breeding tanks and fish ponds water should be allowed to flow through a set of wire netting sieve with mesh of 10, 5, 3 and 1.5 mm size.

4. To prevent the penetration of cercariae - pathogens of eye metacercariosis - with inflowing water, to filter the water with about 20 cm thick layer of sand. Filter should be placed into the inflow drainage after filling the pond.

5. To carry out distribution to other fish farms as soon as the fry reached proper size and when they are not yet affected by specific species of parasites.

6. According to the results of parasitological examination of the fry, a long-term bath in copper sulphate /1.5-2 mg/ against ectoparasitic protozoa and a bath in sodium chloride at a dose of 50 g/l for 3 min at the time of fishing and before distribution of fish are the most effective.

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DERMOCYSTIDIUM INFECTIONS OF CULTURED FISHES

O.N. BAUER

Zoological Institute, USSR Academy of Sciences, Leningrad, USSR

ABSTRACT

A survey of Dermocystidium infection of cultured freshwater fishes is given. It is noted that several species of this genus may act as pathogens causing diseases of gills and skin. There are 5 species infecting carp of young age. The systematic position of Dermocystidium is not clear and features used as species criteria are rather poor. Further investigations are needed to clarify this.

INTRODUCTION

Dermocystidium infections of freshwater fishes have been described for several decades /Reichenbach-Klinke 1950, Scheer 1956, Schulman 1962/ but little is known about their pathogenicity on fish in natural waters and under artificial conditions. Their systematic position was also discussed. For a rather long time they were considered to be Haplosporidia but doubts arose and several protozoologists were of the opinion that the genus Dermocystidium should be excluded from Haplosporidia. Scholtyseck /1979/ in his excellent publication on the fine structure of Protozoa deals with D. marinum infecting the marine shell fish, Crassostrea virginica causing mortality of the host. Scholtyseck described in its spore an apical complex comparable to that found in Sporozoa. This complex consists of a conoid, polar ring up to 39 subpellicular microtubules, rhoptries and micronemes.

Nevertheless, these data are also discussed, because it is said /Schulman, personal communication/ that D. marinum has

nothing to do with Dermocystidium from fishes and is to be excluded from this genus. So the position of Dermocystidium species seems to be still doubtful and an investigation of fine structure of such species should be made to elucidate the problem.

Criteria of Dermocystidium species are also doubtful. Such features as the size of spores, the form of cysts and some others were used, but till now no key for the species of the genus has been proposed.

During the last decade new data have been published showing that Dermocystidium may act as a pathogen causing disease of freshwater fishes. Pronin /1976/ described a case of high infection of young perch, Perca fluviatilis in a lake of Transbaikalian. This species has been found in many water bodies of Europe and Northern parts of Asia, but no data of its pathogenicity has been published. In Pronin's case, D. percae infected young perches /0+, I+/ in several investigated lakes, but only in one the rate of infection of fins and skin, where the parasite is located, was up to 100 %. Such a high rate was observed during 1966-1968, but then it decreased. These fluctuations are connected with the water level of the lake. When it is low the infection decreases, when high - it increases. When the cyst bursts and the spores enter the water, lesions are observed leading to the decrease of growth rate up to 18 %. No mortality has been noted.

A new species, Dermocystidium anguillae causing a gill disease of elvers /juvenile Anguilla anguilla/ has been described by Spangenberg /1975/. Recently Ghittino et al. /1981/ published some data on the infection of elvers cultured in temperate waters. The cysts of D. anguillae were present in about 22 % of examined eels and each affected gill arch harboured up to 18 cysts. Mortality was about 6 % of the cultured fish. It seems that high temperature was the cause of the high infection rate.

A Dermocystidium infection in Atlantic salmon /Salmo salar/ parr has been described in Scotland in the winter of 1977-78 /McVicar and Wotten, 1979/. It is unusual that the parasite is located in the viscera. The visceral cavity of affected fish contains an extensive yellow-white caseous mass consisting of

parasite cells arranged in a series of nodules as large as 1.2 mm in diameter. The spores are 8 mm in size, spherical. This case shows that Dermocystidium species may be parasites not only of gill, skin and fins but also of inner organs. But the infection rate was only 3 % of the affected population.

Several Dermocystidium species were found in wild and cultured carp, Cyprinus carpio. Hoshina and Sahara /1950/ were the first to describe a Dermocystidium from this fish under the name D. koi. This species infects the skin forming round cysts. But the description is rather poor. Allamuratov /1965, in Allamuratov, 1974/ found even two species investigating the wild carp in the river Surkhandarya, the tributary of Amudarya in Central Asia. One species, D. kamilova infects the gills forming cysts up to 1.8 mm in length. The second, D. kobiacovae /the original name is wrong - D. kobiacovi/ is a skin parasite with egg-like small cysts of 0.20x0.15 mm. The round spores of both species are very similar in size. There is no data on the influence of these parasites on the fish.

Lopukhina /1969/ in her carefully prepared studies on gill necrosis of cultured carp in the North-West districts of the USSR described structures very similar to the Dermocystidium cysts. They have been found from the second part of winter and during spring. Such cysts are round and up to 2 mm in diameter. During spring 20-30 small and round spores are formed of 3.0--5.5 µm in size. Their structure resembles that of Dermocystidium spores. Lopukhina thought them to be one of the pathogens of carp gill necrosis.

Some years later Červinka et al. /1974/ found such structures in one-year-old cultured carp in several fish farms of the CSSR. They described the parasite as D. cyprini Červinka et al. 1974, a pathogen causing losses up to 22 % of young carps. Due to their description this species differs from all Dermocystidium species of freshwater fishes known before. Whether D.cyprini is the pathogen of carp gill necrosis is rather doubtful because recently a virus has been described as the real pathogen of the disease. A non-parasitic form of gill necrosis has also been described /Bauer et al. 1981/.

During last years a new infection of carp has been described in the Southern zone of carp culture in the USSR. Its patho-

gen is D. erschovi /Garkavi et al. 1980/, which has been reported from the Ucrainian and Moldavian fish farms /Kulakovskaya, Stryzhak 1980/. D. erschovi forms rather big pustules in the subcutaneous layer of the skin up to 20 mm in diameter. Such pustules contain long sausage- or even thread-like cysts full of spores characteristic for Dermocystidium. When the pustule bursts a lesion is left, which disappears after some time. This infection is observed in one-year-old carp in spring. The infection rate was up to 30 % in different fish farms. The infected fishes had 2-3 pustules each, sometimes more. No mortality was noted. D. erschovi differs from all the other representatives of this genus infecting carp and described in different countries. Its most peculiar feature is the form of cysts and extremely big spores up to 16 µm in diameter. Such big pustules could never be observed again.

The aim of this survey is to draw attention of fish pathologists to Dermocystidium infection of freshwater fishes especially the cultured ones, which is to be characterized in some cases as a real disease influencing growth rate and resulting in fish mortality. As the position of this genus is rather doubtful, investigation of fine structure of all developmental stages is badly needed. Data on pathogenicity and epizootiology were also rather scarce till now, explaining why many manuals on parasitic infections of fish avoid describing this disease.

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ENVIRONMENTAL EFFECTS

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RELATIONSHIP OF WATER QUALITY AND INFECTIOUS DISEASES IN CULTURED CHANNEL CATFISH

J.A. PLUMB

Southeastern Cooperative Fish Disease Project,
Department of Fisheries and Allied Aquacultures,
Alabama Agricultural Experiment Station,
Auburn University, Alabama 36849, USA

ABSTRACT

Channel catfish /Ictalurus punctatus/ is the most extensively cultured food fish in the United States. During the past decade an increase in intensity of catfish culture methods has led to a reduction in water quality which increases stress on the fish and makes them more susceptible to disease. Some of the primary water quality characteristics that influence disease susceptibility are high CO_2, NH_3, and nitrite, and low O_2. Although each of these factors is individually stressful to the fish they reduce disease resistance more significantly in combination. An example of a case involving an O_2 depletion as result of an algal die-off, followed by an Aeromonas hydrophila infection, is described. The relationship of water temperature and alkalinity on disease susceptibility is also discussed. Polyculture utilizing silver carp, tilapia, and grass carp as herbivores in combination with channel catfish to help improve water quality is discussed.

INTRODUCTION

Since 1970 the channel catfish /Ictalurus punctatus/ has become the most widely cultured commercial freshwater food fish in North America. Because of its cultural requirements for warm water, the vast majority of channel catfish are grown in the southern United States. Farm production increased from 2.8 million kg raised in 20,000 hectares in 1970 to approximately 44

million kg raised in 28,000 ha in 1980. Although some catfish are raised in raceway units /about 4 %/, the vast majority /96 %/ of the production are raised in intensively or extensively managed static ponds.

To improve production efficiency per culture unit, farmers have increased pond stocking rates tremendously during the last 10 years. In 1965 it was recommended that catfish be stocked at rates not to exceed 4,000 fish per hectare and feed not exceeding 30 kg per hectare per day, thus producing approximately 2,000 kg per hectare per year. At present, most farmers are producing a minimum of 5,000 kg of fish per hectare per year; some are obtaining yields as high as 10,000 kg per hectare. This requires feeding rates of 80 to 200 kg per hectare per day. Consequences of these rates of production are often catastrophic. As feeding rates increase, nutrients accumulate, algal blooms grow at a rapid rate, and oxygen and other water quality problems are frequent. As a result of these water quality and environmental problems, the importance of infectious diseases becomes paramount. Some of these diseases involve the well known pathogens: motile aeromonads, myxobacteria, and parasitic protozoa. However, stressful conditions are also leading to disease conditions with which we are not familiar. The objective of this paper is to describe the water quality and environmental conditions that affect infectious disease susceptibility in catfish culture ponds. A second objective is to discuss the potential of polyculture of channel catfish and herbivorous species to improve water quality.

CHEMICAL AND PHYSICAL CHARACTERISTICS

In 1974 a catfish pond on the Alabama Agricultural Experiment Station at Auburn University had severe oxygen depletion because of an algal die-off /Boyd et al. 1975/. Within 3 days of planktonic die-off, O_2 concentration plunged to less than 1 mg/liter, the pH fell from 9 to 6.5, CO_2 rose to 13 mg/liter, and ammonia /NH_3/ increased to 1.7 mg/liter /Figures 1 and 2/. Although most fish survived, recovery was slow and 5 days after the algae die-off many fish in the pond developed hemorrhagic skin lesions and necrotic musculature /Plumb et al. 1976/. Initially these lesions were aseptic, but on the day following their

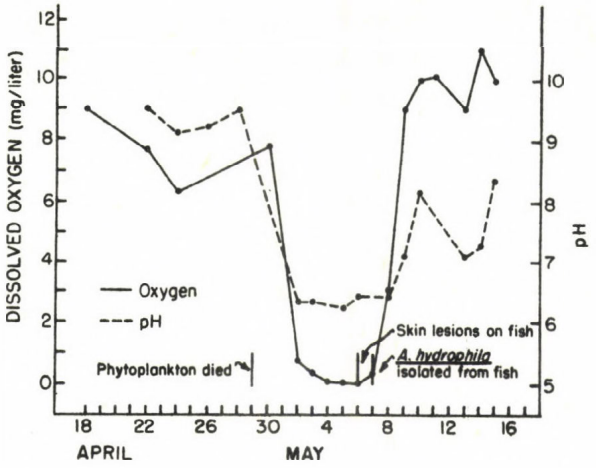

Figure 1 Oxygen concentration and pH in a channel catfish pond before and after a phytoplankton die-off which led to a fish kill and bacterial infection. /Reprinted from J. of Wildlife Disease, Plumb et al. 1976/

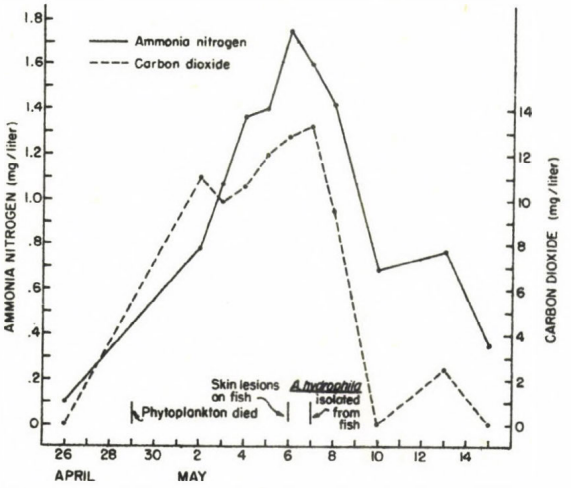

Figure 2 Ammonia nitrogen and carbon dioxide concentrations in a channel catfish pond before and after a phytoplankton die-off which led to a fish kill and bacterial infection. /Reprinted from J. of Wildlife Disease, Plumb et al. 1976/

first appearance, the facultative bacterium Aeromonas hydrophila was isolated from lesions and internal organs. When water quality improved /O_2, CO_2, NH_3, and pH returned to pre-die-off levels/ the incidence of lesions dropped drastically. It was postulated that during the hypoxic conditions, muscle tissues and internal organs were deprived of adequate oxygen, which resulted in tissue death and necrosis. This was followed by necrotic epithelium and musculature that was secondarily invaded by A. hydrophila.

The foregoing example serves to illustrate the dynamic triad of host-pathogen-environment in epizootiology and fish health. Water quality is of extreme importance in the health of cultured catfish. Management practices that are designed to maximize productivity do little to improve environmental quality. With increased environmental degradation, the fish's resistance to disease decreases. A few potential fish disease-causing organisms are obligate pathogens /examples are Ichthyophthirius and channel catfish virus/, which are not normally found in pond water. Most catfish disease-causing organisms are facultative pathogens /Aeromonas, Pseudomonas, Edwardsiella, and Myxobacteria/ and are commonly found in most waters. Often only the fish's resistance, which is influenced by the environment, stands between a healthy and a diseased fish.

The oxygen depletion phenomenon previously described led to experiments dealing with the effects of O_2, CO_2, NH_3, and temperature on the disease susceptibility of channel catfish to bacterial infections and the fishes physiological response to these factors. Scott and Rogers /1980, 1981/ studied the effects of prolonged sublethal hypoxia on histological and hematological changes in subadult channel catfish that were not encumbered by infection. They found that hematocrit, plasma protein, red blood cell volume, total erythrocyte counts, and total differential leukocyte counts were not sensitive to change during prolonged hypoxia. However, hemoglobin, plasma glucose, and plasma lactic acid of hypoxia-stressed fish deviated significantly from control fish. Spleen, liver, anterior and posterior kidneys, and gills had histopathological lesions due to hypoxia. Gill tissue was most severely affected. After 5 days in oxygenated water,

some of these histological lesions still persisted, indicating that hypoxia-stressed fish do not recover rapidly, thus prolonging their disease susceptibility.

Walters and Plumb /1980/ studied the relationship of O_2, CO_2, and NH_3 concentrations on channel catfish susceptibility to A. hydrophila /Figure 3/. They found that the combination of

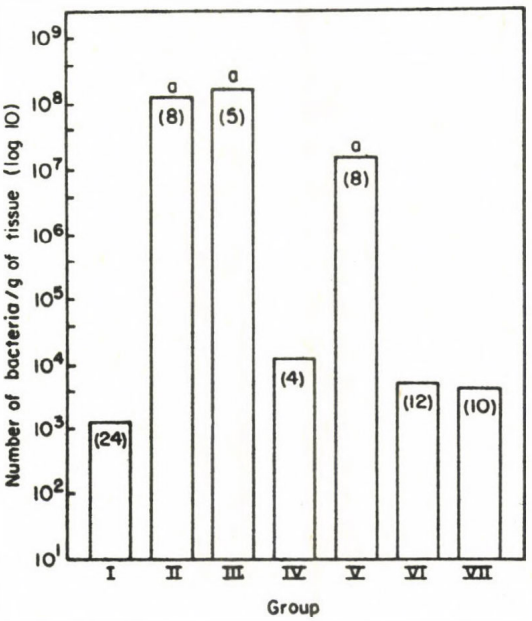

Figure 3 Number of bacteria /all species/ per gram of trunk kidney from channel catfish held in various environmental treatments. I-Low dissolved oxygen /DO/ only; II-Low DO and injected with Aeromonas hydrophila; III-Low DO, injected with A. hydrophila and added NH_3; IV-Low DO, injected with A. hydrophila and added CO_2; VI-Reaeration and injected with A. hydrophila. VII-Noninjected controls held in reaerated water. Treatments that produced numbers of bacteria per gram that are significantly higher /$p < .01$/ are designated by "a". The number of fish sampled is in parentheses inside of the respective bar. /Reprinted from J. Fish Biology, Walters and Plumb 1980/.

1.5 mg/liter of O_2, 1.2 mg/liter of NH_3, and 6.5 mg/liter of CO_2 significantly increased bacterial susceptibility over each individual stressor condition alone. The combination of high CO_2 and NH_3 similarly had a more deleterious effect than low O_2 alone. Not only were stressed fish more susceptible to A. hydrophila,

the incidence of Edwardsiella tarda increased from 3 in control fish to 50 in stressed fish.

Accumulation of un-ionized ammonia /NH_3/ in fish ponds is a serious problem that may reduce disease resistance /Colt and Armstrong 1979/. The specific relationship of NH_3 to fish disease susceptibility probably has to do with impairment of hemoglobin binding with O_2 /Brockway 1950/. High NH_3, among other environmental factors such as low O_2 and crowding, is associated with bacterial gill disease of trout /Burrows 1964/. Ammonia concentration affects the susceptibility of channel catfish to Aeromonas sp. and other bacterial infections /Thune 1975; Walters and Plumb 1980/. Toxic levels of NH_3 range from 0.6 to 2.0 mg/liter, but this depends upon the pH, CO_2 and O_2 concentrations, and fish species /Boyd 1979/. Ammonia's effect on infectious disease susceptibility increased when CO_2 was high, and O_2 and pH were low /Walters and Plumb 1980/.

Accumulation of nitrite in pond water results in methemoglobinemia, also known as "brown blood disease" /Colt and Armstrong 1979; Huey, Simco and Criswell 1980/. The incidence of methemoglobinemia in channel catfish is particularly high during the fall and spring. Nitrite oxidizes the hemoglobin in red blood cells forming methemoglobin which gives blood the brown color. Methemoglobin has no oxygen carrying capacity so that the fish's capacity to extract oxygen from the water is reduced. Whether or not "brown blood" leads to infectious diseases is unknown, but mortalities are chronic rather than acute in fish populations with this condition.

Dissolved oxygen concentration is probably the most important water quality characteristic in culture ponds, however, it is difficult to discuss O_2 without including most other water quality factors. There is little doubt that chronically low O_2 concentrations interfere with physiological performance /Warren et al. 1973/. Reduced physiological performance will also depress the fishes' resistance to infections. Therefore, within 10 days of an oxygen depletion stress, fish will typically develop some type of disease process, often bacterial. The example discussed earlier typifies the relation between O_2 concentrations and the other critical water quality parameters. First,

the algae, or other plants, die thus stopping photosynthetic O_2 production. As the vegetation decomposes, O_2 concentration is reduced, CO_2 is released, and ammonia accumulates. In a catfish pond it is difficult to have one of these conditions without the others and they are more stressful in combination than individually.

Elevated carbon dioxide levels in fish ponds interfere with O_2 binding to hemoglobin in the fish's blood /Basu 1959/. Channel catfish can withstand CO_2 concentrations in excess of 10 mg/liter as long as oxygen concentrations are high /Boyd 1979/. Concentrations of 25 mg CO_2 per liter have been noted in highly enriched catfish ponds during afternoon hours when O_2 levels were also high, particularly during winter, causing an intoxicating effect. Under these conditions fish are stressed and may subsequently become infected with pathogenic organisms.

Although water temperature is usually not considered part of water quality, it is an integral factor in fish disease susceptibility. In most instances a given species of fish has a range of temperatures in which it reproduces best and grows fastest. This is usually the temperature where natural and acquired resistance is the highest. When the fish is exposed to adverse temperatures, either above or below this optimum, disease become more prevalent. Tilapia sp. are good examples of fish living best at temperatures above 21°C. As the temperature drops below 21°C disease susceptibility increases. Trout, on the other hand, have an optimum range of 10 to 16°C and as the temperature rises to 20°C they become more susceptible to a variety of diseases. Fortunately, channel catfish have a wide range of temperature tolerance from 10°C to above 30°C, however, the optimum is from 25 to 33°C. The optimum temperature for most diseases of channel catfish is from 20 to 30°C, although they do become diseased with viral, bacterial, or parasitic organisms at all temperatures. For example, Ichthyophthirius is most severe at 20 to 22°C, but "Ich" infections also may occur at 5 to 7°C but seldom above 23°C. Channel catfish virus only occurs above 23°C and columnaris disease /Flexibacter columnaris/ will usually developabove 20°C in warm water fish. Quines /1978/ demonstrated an increase in mortality of columnaris infected catfish from 15 % at 20°C to 95 % at 30°C.

Rasheed /1978/ demonstrated that channel catfish fingerlings are more susceptible to A. hydrophila at 25°C to 30°C. Below 25°C the virulence of the bacterium is reduced. She also showed that the temperature of the fish's environment is much more important to the fish than to the bacterium. In other words, mortality caused by A. hydrophila during temperature fluctuation is because of the effect of temperature on the fish's physiological and immunological status rather than on the virulence of the pathogen.

Water alkalinity has an effect on the health and disease susceptibility of fish. Bacterial kidney disease /Renibacterium salmoniarum/ outbreaks in trout are more frequent in soft water /< 50 mg/liter as $CaCO_3$/ than in hard / >100 mg/liter as $CaCO_3$/ /Warren 1963/. Although definitive studies to determine the effects of water hardness on the disease susceptibility of channel catfish have not been done, general observations indicate that fewer disease problems occur in hard water.

When discussing the relationship of water quality to fish disease, it is virtually impossible to concentrate on only one parameter. Ammonia toxicity is affected by pH; CO_2 toxicity is affected by O_2 concentration; temperature affects O_2 and metabolism, and so on. Water quality is as dynamic and complex as fish disease resistance in that it is perpetually changing. It is not sufficient to concentrate on one aspect and ignore the other.

POLYCULTURE AND WATER QUALITY

It has been pointed out that water quality, more specifically oxyger concentration, is an important aspect of the epizootiology of many diseases of monocultured channel catfish in the United States. One of the major contributing factors to the poor oxygen levels and water quality is the enormous algal blooms that develop as a result of application of large quantities of high protein feeds to fish ponds. These algal blooms should be controlled through chemical or biological means, preferably the latter. Polyculture, using herbivorous species in conjuction with the channel catfish to improve water quality, has been investigated by Behrends /1977/ and Dunseth /1977/. Various combinations of channel catfish, silver carp /Hypophthalmichthys

molitrix/, grass carp /Ctenopharyngodon idella/, and monosex tilapia /Sarotherodon sp./ were used to control algal blooms and provide a more suitable water quality for catfish culture.

Behrends /1977/ concentrated on the effects of channel catfish, silver carp, grass carp, and tilapia on primary productivity, daily community respiration values, diversity of phytoplankton, and dissolved oxygen concentrations. Ponds were stocked at 7,400 channel catfish, and/or 1,975 tilapia, and/or 2,479 silver carp per hectare. Ponds were also stocked at 11,100 channel catfish per hectare with the same numbers of tilapia and silver carp. All treatments had 50 grass carp per hectare.Polyculture of catfish, at the lower rate in combination with silver carp, was more desirable than monoculture of catfish. These ponds had consistently higher early morning and late evening dissolved oxygen concentrations and required less supplemental aeration.

Dunseth /1977/ evaluated the relationship of water quality to different stocking rates in monoculture channel catfish compared to polyculture of catfish, silver carp, and male tilapia. Water quality was improved in polyculture with channel catfish and silver carp over monoculture catfish. During September, mean weekly oxygen concentrations were significantly higher in treatments with silver carp.

In each of these studies, undesirable blue-green algae were eliminated by silver carp. In general, phytoplankton densities were significantly lower /$P < .05$/ in polyculture with silver carp than in monoculture catfish populations. Neither of these studies evaluated the health status of the fish populations. However, considering the relationship of phytoplankton densities and accumulation of organic matter in the water, reduced oxygen concentration, and increased disease incidence, it follows that polyculture using silver carp with catfish may be a useful managerial and biological tool to control algae and reduce disease production losses. Improved oxygen profiles and improved water quality will reduce environmental stress on the primary culture species, channel catfish, resulting in fewer disease problems.

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TRANSAMINASE ENZYME ACTIVITY OF CYPRINID FISH DEPENDING ON ENVIRONMENTAL FACTORS AND BACTERIAL INFECTION

G. JENEY, J. NEMCSÓK*, J. OLÁH

Fisheries Research Institute, Szarvas
*Department of Biochemistry, József Attila University, Szeged, Hungary

ABSTRACT

In human diagnosis the detection of injuries of different tissues /liver, kidney, muscle/ by determining the transaminase activity /GPT: glutamic acid-pyruvic acid transaminase; GOT: glutamic acid-oxalacetic acid transaminase/ in blood is a commonly used and well-proved method. In our work this method was used to detect damage of tissues of fish caused by adverse environmental factors and bacterial infection. As a first step serum GOT and GPT activities were determined in three fish species /Hypophthalmichthys molitrix, Ctenopharyngodon idella, Cyprinus carpio/ depending on their ages. In all the investigated species the highest enzyme activity was measured in the adult, three-year-old specimens. There were no significant differences between serum transaminase activity in different species of the same age.

Furthermore, serum transaminase activity of carp was measured as a function of water temperature, seasons, different NH_3 concentrations and bacterial infection. GOT and GPT activities changed parallel with the increase of water temperature. The highest activity was measured during the summer-period /May--August/ while the lowest in winter. This might correlate with the metabolic rate as an effect of higher water temperature, i.e. it might reflect the increased ammonia excretion and detoxication. Serum transaminase activity was enhanced together with increased ammonia concentration and with the duration of the treatment reflecting the role of these enzymes /GOT, GPT/ in ammonia detoxication. This could indicate gill necrosis fol-

lowing ammonia treatment or bacterial infection. According to our results the determination of serum transaminase activity in fish proved to be a suitable method for detecting tissue damage caused by adverse environmental factors and bacterial infection.

INTRODUCTION

In human diagnosis the detection of injuries of liver, kidney and muscle tissues by determination of transaminase activity in blood is a routinely used and well-proved method. Under normal conditions transaminases are located in cytoplasm and mitochondria. Damages and lysis of cells result in relatively high quantities of these enzymes in blood.

In our work the serum GOT and GPT activity in three fish species /common carp, silver carp and grass carp/ was determined. Furthermore, serum transaminase activity was measured in carp as the function of water temperature, seasons, different NH_3 concentrations and bacterial infection.

MATERIALS AND METHODS

For measuring age-dependent serum transaminase activity of fish, 1, 2 and 3 year-old specimens of common carp, silver carp and grass carp were used. To determine the seasonal- and water temperature-dependent activity of transaminases, 1 and 3 year-old specimens of common carp and silver carp were used. Measurements were made at least once a month in every season.

The effect of 0.1, 0.4 and 1.0 ppm NH_3 on transaminase activity was measured on the 8th, 14th, 20th and 24th days, after each treatment. Blood samples were taken both from treated and control animals. Enzyme activity was measured after centrifugation from the haemolysis-free serum.

The given values are the averages of measurements in at least 10 fish. Enzyme activity was measured photometrically by means of VSU-2 Spectrophotometer using Boehringer KIT-s.

Determination of GOT and GPT activities

Reaction mixture for GOT: 0.25 ml 0.1 M phosphate buffer /pH 7.4/ containing 0.1 M L-aspartate and 2 mM α-ketoglutarate

+ 0.050 ml blood serum /0.050 ml dist. water in blank/. Reaction mixture for GPT: 0.25 ml 1.0 M phosphate buffer /pH 7.4/ containing 0.2 M DL-alanine and 2 mM α-ketoglutarate + 0.050 ml blood serum /0.050 ml dist. water in blank/. After incubation for 60 min /30 min for GPT/ at 37°C, 0.25 ml 1 mM 2,4-dinitrophenyl--hydrazine was added to each sample and the mixture was incubated for 20 min at 20°C. After the addition of 2.5 ml 0.4 M NaOH solution, absorbance was measured at 540 nm.

RESULTS AND DISCUSSION

Serum transaminase activities of common and silver carp increased as a function of age of the fishes - except for 1-year--old carp. There were no differencies, however, in enzyme activities regarding grass carp of different ages /Table 1/.

Table 1 Changes of serum transaminase /GOT and GPT/ activities of carp, silver carp and grass carp depending on the ages of fishes. Values are the averages of at least 10 fishes /±S.D./. Enzyme activity is expressed as U/l

	GOT	GPT
Silver carp$_3$	35.64±7.29	2.36±0.09
Silver carp$_2$	19.99±5.13	1.69±0.23
Silver carp$_1$	14.04±4.30	0.82±0.17
Grass carp$_3$	16.57±3.58	1.00±0.08
Grass carp$_2$	21.94±2.37	1.00±0.20
Grass carp$_1$	18.99±3.00	1.02±0.14
Common carp$_3$	38.42±4.47	2.79±0.74
Common carp$_2$	30.84±8.32	2.55±0.19
Common carp$_1$	29.06±4.98	8.16±0.36

Transaminase activity showed different values depending on environmental factors. GOT and GPT activities were the highest in summer in both fish species /carp and silver carp//Table 2/.

Higher water temperature caused increased transaminase activities in one-year-old carps. The maximum values were measured at 27°C /Table 3/.

Table 2 Seasonal alteration of serum transaminase /GOT,GPT/ activities of common and silver carp. Values are averages of at least 10 fish /±S.D./. Enzyme activity is expressed as U/l

	GOT	GPT
Common carp$_3$		
April	21.43±4.31	1.03±0.06
May	30.32±4.21	1.52±0.22
July	25.43±6.10	2.88±3.02
August	38.42±4.47	2.79±0.74
September	19.20±2.72	2.43±0.49
October	29.35±3.53	1.93±0.78
February	18.46±3.87	4.97±1.34
Silver carp$_3$		
April	19.99±5.13	1.69±0.23
August	32.64±8.66	1.47±0.45
October	17.40±4.46	2.27±1.06
February	20.30±3.45	3.05±0.97

Table 3 Changes of serum transaminase /GOT, GPT/ activities of carp depending on water temperature. Water temperature was increased step by step /5°C - a/ and /10°C - b/. Values are averages of at least 10 fishes /±S.D./. Enzyme activity is expressed as U/l

	GOT	GPT
a/ 5°C	14.22±2.34	2.70±0.72
10°C	14.91±2.92	3.33±0.72
15°C	22.09±3.48	4.06±0.89
20°C	26.71±3.47	3.48±0.42
b/ 7°C	18.04±3.71	3.39±1.12
17°C	10.07±1.22	5.93±1.93
27°C	61.52±0.54	11.30±1.31

Table 4 Effect of NH_3 in different concentrations /0.1, 0.36, 0.8 ppm/ and bacterial infection* on serum transaminase activity of carp in the course of time. Values are averages of 8-16 fishes /±S.D./. Enzyme activity is expressed as U/l.

NH_3 concentration	GOT	GPT
Start of experiment	7.72±2.68	2.27±1.76
Control		
8th day	7.86±1.45	0.39±0.18
14th day	11.15±1.00	1.79±1.26
20th day	10.16±2.15	3.18±0.82
24th day	24.05±5.16	4.31±1.56
0.1 mg/l NH_3		
8th day	10.04±2.72	0.72±0.59
14th day	11.59±1.34	1.65±0.39
20th day	11.89±2.47	1.92±1.56
24th day	22.64±4.40	4.85±1.56
0.36 mg/l NH_3		
8th day	11.76±5.37	1.73±0.67
14th day	17.53±2.16	2.12±1.52
20th day	14.78±4.97	2.32±1.36
24th day	27.45±3.29	5.29±1.63
0.8 mg/l NH_3		
8th day	16.32±2.36	6.22±0.73
14th day	24.28±6.30	1.91±0.18
20th day	13.42±2.48	2.34±1.35
24th day	25.00±7.76	4.82±2.50

*Infection was performed on the 20th day after measurement

The highest NH_3 concentration /1.0 ppm/ increased GOT activity during the treatment, but the change of GPT was not significant. However, it is remarkable that serum GOT and GPT activities increased significantly even at low NH_3 concentration after bacterial infection /Table 4/.

Many articles have dealt with the role of transaminases in tissues damaged by environmental pollution where the tissue damaging effects of several organic chemicals were detected by measuring serum transaminase activity /Bell 1968, Reichenbach-Klinke 1972, McKim et al. 1970/. According to our results, adverse environmental factors /highly increased NH_3 in the water, bacterial infection/ can cause tissue necrosis as well, and these processes can be followed by controlling serum transaminase activity of fish.

Therefore, normal serum transaminase activity values of the investigated fish species should be determined as a function of age of fish, season and microenvironmental factors. No regular measurements of this type have been performed yet because of the lack of generally accepted methods. These measurements were proposed by other authors as well /d'Appolonia and Anderson 1980, Kristofferson et al. 1974/. The changes of transaminase enzymes in different seasons of the year and at different water temperatures might correlate with the fact that the metabolic rate of poikilotherm animals strongly depends on the temperature of their environment. So the fish tolerate the increasing concentration of NH_3 to a certain extent as a consequence of increased water temperature - by HCl increased transaminase activity. The increase of serum transaminase activity after treatment with NH_3 of gradually increasing concentration might reflect that, these enzymes are involved in the detoxication of ammonia /Nemcsók et al. 1980/ but it might be a consequence of gill necrosis, as well.

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EFFECT OF NH_3 ON BLOOD GLUCOSE AND CATECHOLAMINE LEVEL, GOT, GPT, LDH ENZYME ACTIVITY AND RESPIRATION OF FISHES

J. NEMCSÓK, K. GYÖRE*, J. OLÁH*, L. BOROSS

Department of Biochemistry, József Attila University, Szeged,
*Fisheries Research Institute, Szarvas, Hungary

ABSTRACT

The blood sugar- and catecholamine level, LDH /lactic-acid--dehydrogenase/, GOT /glutamic acid-oxalacetic acid transaminase/, GPT /glutamic acid-pyruvic acid transaminase/ activities and the respiration of carp, silver carp and sheatfish /weighing 5-15 g/ were investigated following the treatment with NH_3 of different concentrations.

A correlation was observed between the NH_3 content of the water and the intensity of O_2 consumption regarding the three species.

Ammonia - as a toxic agent - caused stress in fishes by changing their metabolic processes as it was reflected by the enhanced blood sugar and catecholamine level of the blood sera. Higher NH_3 concentration induced anoxia in the tissues.

The physiologically toxic NH_3 level is neutralized in the organism by normal detoxical processes, more exactly by means of increased GOT and GPT activities.

INTRODUCTION

Ammonia toxicity is reputed to be one of the main causes of death in fish culturing /Hampson 1976/. In mammals most nitrogen is excreted as urea, uric acid or creatine. In fishes nitrogen is generally excreted directly as ammonia without a detoxicative mechanism. In mammals normal plasma ammonia concentration is under 1 ppm and 5 ppm is fatal /Baldwin 1948/. In fish, normal NH_3 concentrations in the plasma depend on the family or genera and

sometimes on species /Robertson 1954, Lloyd 1961, Burrows 1964/. The specific biochemical mechanism of ammonia toxicity is unknown, but it has been associated with alterations in carbohydrate and amino acid metabolism /Clifford et al. 1972/. The concensus is that acute ammonia intoxication deranges intermediary metabolism primary to ATP production, resulting in depletion of energy stores especially in the brain, localized in the brainstem /Schenker et al. 1967/.

Smart /1976/ investigated the possibility that ammonia may produce severe changes in gill's structure and at acutely lethal concentration, found relatively minor histopathological changes. Ammonia toxicity may inhibit the ability of haemoglobin to combine with oxygen. Acute ammonia poisoning induce permanent stress in fishes with changes in plasma glucose and lactate level which adversely affects the physiological and biochemical processes. In clinical medicine, serum enzyme analysis has been used for decades to diagnose both the site and extent of organ injury. In the field of environmental toxicology, serum enzyme analyses are becoming increasingly important for the toxic effect of chemical pollutants. Two serum enzymes, frequently used to diagnose sublethal damage to different organs /liver, gill, muscle/ by pollutants are glutamate oxaloacetate transaminase /GOT/ E.C.2.6.1.2 and glutamate pyruvate transaminase /GPT/ E.C.2.6.1.1 /Bell 1968, Mehrle and Bloomfield 1974, Malevski and Montgomery 1974, Racicot et al. 1975/. These two transaminases, along with glutamate dehydrogenase, play an important role in ammonia detoxification in freshwater teleosts /Hochachka and Somero 1973/. Thus the aim of this paper is to obtain further information regarding the damaging effects of NH_3 toxicity in three fish species.

MATERIAL AND METHODS

Catecholamines were determined according to the method of Anton and Sayre /1962/.

The rate of oxygen consumption of test-animals was measured in a flow-through respirometer /Fig. 1/. To reduce alterations in the reproducibility of oxygen level measurements by microorganisms, streptomycin and chloromycetin was dissolved in the test-water in a final concentration of 32 $mg.l^{-1}$ and 25 $mg.l^{-1}$, res-

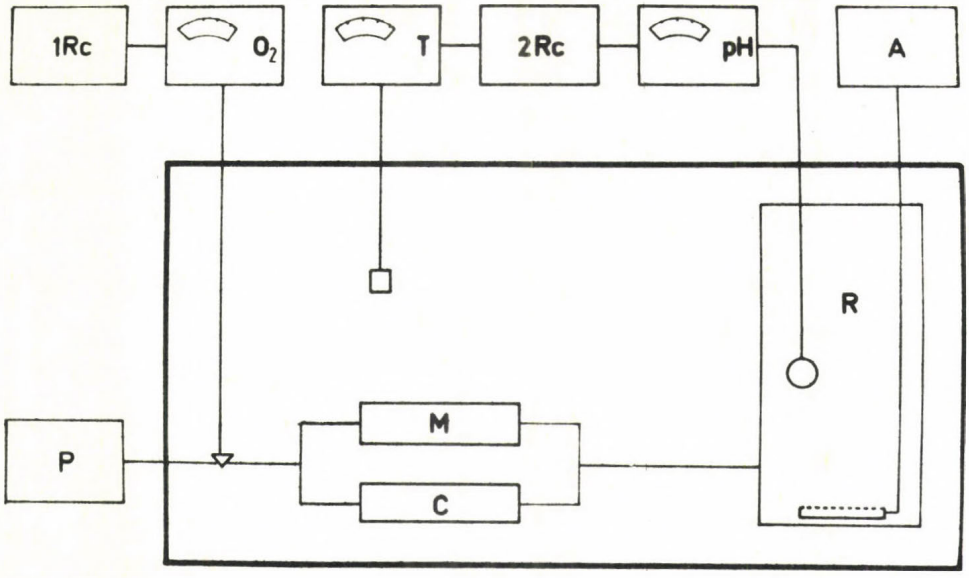

Figure 1 Respirometer /A: air; R: reservoir; P: peristaltic pump; O_2: measuring of oxygen; T: measuring of temperature; pH measuring of pH; 1-2 Rc: potentiometric rerecorder; M: measuring box; C: control box/

pectively. The pH of water was adjusted by phosphate-borate buffer solution to 8.3 value. Ammonia levels corresponded to appropriate NH_4Cl concentrations. Water temperature was held constant at 25 $\pm$0.1 ^{o}C throughout the experiments. To the respirometer immersed into temperated water-bath, oxygen saturated water 8.18 mg.l^{-1} was supplied at constant flow rates using peristaltic pumps. Oxygen concentrations were measured by means of a YSI Model 53 oxygen monitor /Yellow Springs Inc., Yellow Springs, OH, USA/. Rates of oxygen consumption /R/ were calculated by using the equation of Winberg:

$$R \text{ /mg } O_2 \text{ /h/ ind / } = a\, W_t^b$$

where W_t = body weight.

Blood sugar level, LDH, GOT and GPT enzyme activities were measured by using standard assay kits /Boehringer, Mannheim, FRG/.

RESULTS AND DISCUSSION

In case of carp, sheatfish and silver carp - contrary to the changes of blood sugar level - the serum adrenaline level increased at the highest ammonia concentration /1 mg/l/ only. There were no significant changes regarding the serum noradrenaline level /Table 1/.

Table 1 The effect of different concentrations of NH_3 on the adrenaline level of blood serum in carp, silver carp and sheatfish. /Values expressed: g adrenaline/ml serum/

Species	Ammonia concentration			
	Control	0.2 mg/l	0.6 mg/l	1.0 mg/l
Carp	0.041	0.038	0.048	0.059
Silver carp	0.061	0.046	0.051	0.078
Sheatfish	0.038	0.048	0.045	0.050

The enhanced blood glucose and adrenaline level reflected the general stress effect in fishes caused by ammonia. However, the increase of serum adrenaline level was less significant than that of the blood glucose. This might be due to the insufficient sensitivity of the method of adrenaline determination. So the inaccurate measurements might cover the real differences between the control and the low ammonia concentration treated samples.

Specific changes were observed for each of the three investigated fish species in the respiration when the ammonia levels were increased /Fig. 2/.

For common carp and silver carp, respiration decreases immediately at increasing NH_3. For the sheatfish respiration is stimulated up to 0.25 mg . l^{-1} NH_3 /un-ionized ammonia/ but later decreases rapidly similarly to the above cyprinids. Increased blood-sugar levels at increasing un-ionized ammonia levels suggest stress situation. The ammonia-dependent respiration and blood sugar level curves are inverse for each investigated species /Fig. 3/. "Anoxic condition" at tissue level can be deduced from the parallel run of blood-sugar level and LDH curves which correspond to the higher rate of glycolysis. There was a strong interrelation between decreased O_2 consumption and in-

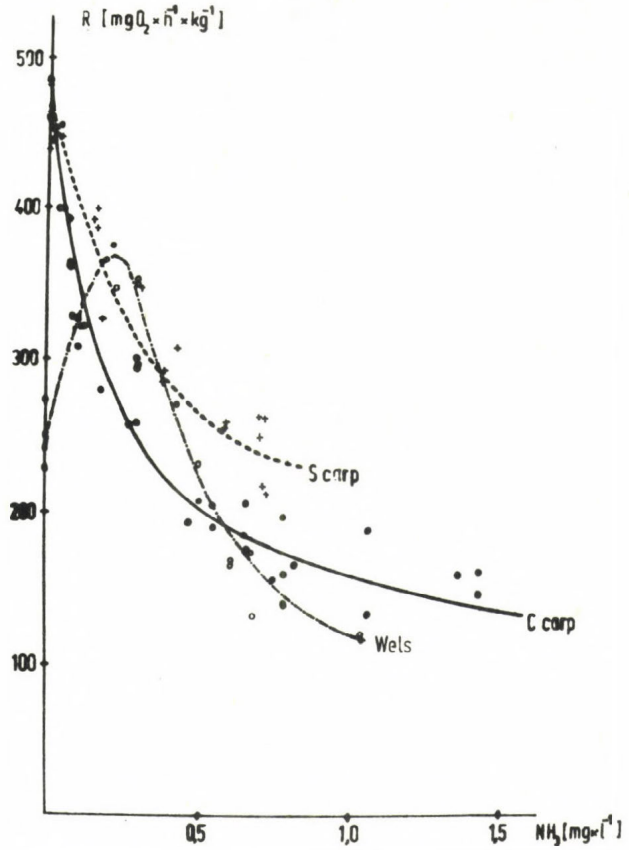

Figure 2 Oxygen consumption of the three fish species in the function of ammonia /NH_3/ content of the water

creased LDH activity and blood sugar level in each investigated species.

This might be due to the lack of O_2 in different tissues as a result of decreased respiration. The increased LDH activity and blood glucose level indicated metabolic changes induced by stress; the catabolism of glucose moved towards the production of lactic acid, which is very dangerous and toxic to fishes /Nakono and Tomlinson 1967/.

The high rate of LDH activity in sheatfish /Fig. 3c/ as compared to cyprinids refers to the greater importance of anaerobic processes.

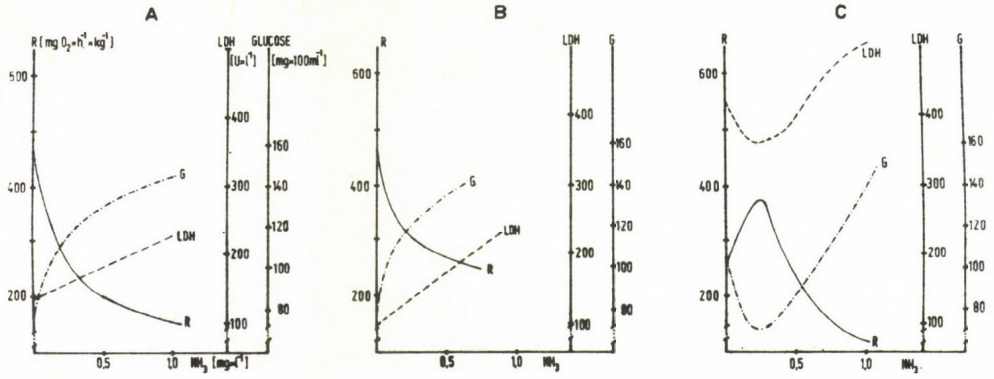

Figure 3 Oxygen consumption of three fish species and change of blood glucose level and LDH enzyme activity in the function of ammonia /NH_3/ content of water. /A: common carp; B: silver carp; C: sheatfish; Respiration: ——; LDH: -----; Glucose: -·-·-·-/

High environmental level of NH_3 inhibits the release of ammonia, the end-product of protein catabolism /auto-intoxication/. The ammonia detoxication through the GOT and GPT transaminases consumes a substantial amount of α-ketoacids from: tricarboxylic acid cycle /Krebs-cycle/, thus decreasing the energy supply of the cells especially of the neurons. In the case of carp and silver carp GOT activity increased slightly at higher NH_3 levels only, but GPT activity increased at lower NH_3 concentration range as well /Fig. 4/.

In the blood serum of sheatfish GOT activity did not increase even at 1 ppm NH_3 treatment, but GPT activity showed a slight increase. From the experiments we concluded that NH_3 detoxication takes place first by the GPT, but at higher NH_3 concentration GOT is involved in this process as well. Common carp has the highest rate of detoxication as well as ammonia tolerance among the three fish species investigated. Presumably transaminase enzymes enter the detoxification only when fish are already unable to excrete NH_3 through the gill by passive diffusion. Several papers reported damage of liver, glomerular atrophy and epithelial necrosis of gill in some organic and inorganic chemical-exposed fishes /Bell 1968, Kristofferson et al. 1974,

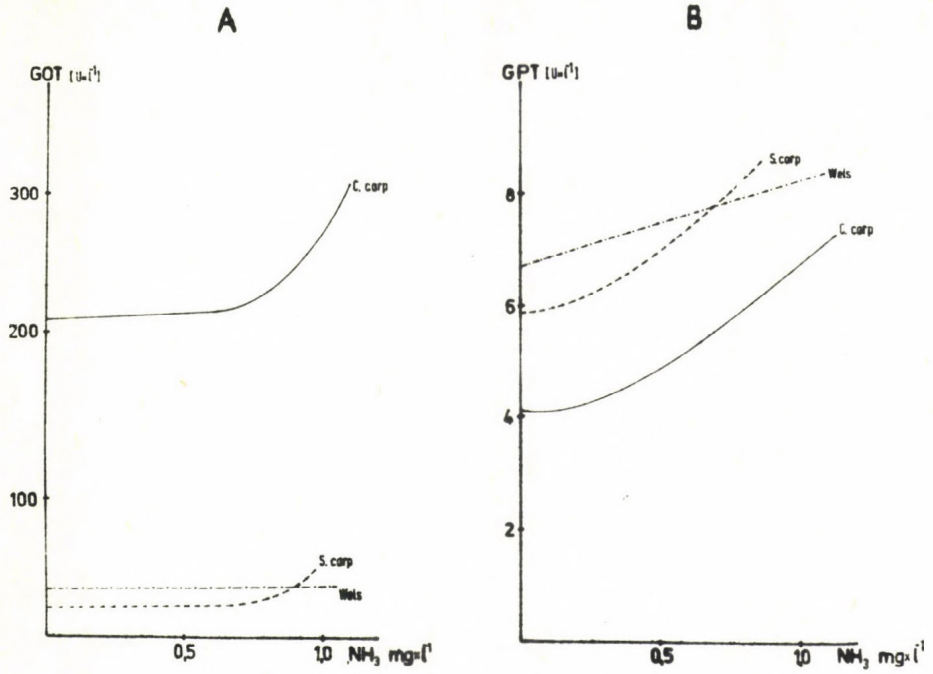

Figure 4 Changes of GOT /A/ and GTP /B/ activities in the function of ammonia /NH_3/ content of water

Reichenbach-Klinke 1972/. Therefore, the elevated GOT and GPT activities in carp and silver carp serum might reflect the damage of liver but the damage of other organs - kidney, and/or gills - is also possible.

The enhanced blood glucose level and LDH activity showed that NH_3 could produce stress in fishes. Permanent stress includes impaired γ-globulin formation and depressed interferon production, which play an important role in the resistance of fishes to various bacterial and viral diseases.

SUMMARY

Oxygen consumption, blood-sugar level, LDH, GOT and GPT activities in tissues of three fish species /Cyprinus carpio L., Hypophthalmichthys molitrix Val., Silurus glanis L./ exposed to sublethal and lethal ammonia concentrations were monitored or measured.

The results show that the un-ionized ammonia even at low level

- depresses the respiration rate of fishes,
- creates a stress situation with partial anoxia at tissue level,
- inhibits the passive ammonia release,
- increases the activity of ammonia detoxicating transaminases /GOT, GPT/,
- produces a competition for energy between respiration and detoxication.

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HISTOPATHOLOGICAL DIFFERENTIAL DIAGNOSIS OF GILL CHANGES WITH SPECIAL REGARD TO GILL NECROSIS

É. KOVÁCS-GAYER

Central Veterinary Institute, Budapest, Hungary

ABSTRACT

The results of histological examinationsin 1979 and 1980 on gill necrosis have been summarised in this work. In the pathogenesis of gill necrosis, acute and chronic inflammatory stages were established that may develop into diffuse necrosis. By histological examination, data were gained for differential diagnosis of gill necrosis of carp. It was stated that histopathology is important in the diagnosis of the disease, especially at early stages. But the fact must also be considered that anoxia, ammonia toxicosis and the effect of pesticides, heavy metal-ions can also be the cause of acute inflammation in the gill.

Pathology of the disease is often aggravated by parasites and bacteria as Flexibacter spp.

INTRODUCTION

Gill necrosis is a common disease not only in Hungary but in other European countries as well. Etiology of the disease is not clear. Some authors consider infective agents /virus, bacteria/ to be the cause of the disease. According to Schreckenbach and his co-workers gill necrosis is an ammonia in- or autotoxication depending primarily on environmental factors /Schreckenbach et al. 1975/. The diagnosis of gill necrosis has been dealt with at length but there are only few data on the histopathology of the disease /Lopuchina 1969/.

The summary of changes in the gill is given in Eller's work. The causes under discussion can be very different: physical factors /anoxia, pH, high or low temperature/, chemical ones /NO_2,

NO_3, NH_3, heavy metals, pesticides, etc./, feeding abnormalities, deficiency diseases /e.g. pantothenic acid/, errors in technology, and infective agents /viruses, bacteria, fungi, parasites/ /Ghittino 1969, Bootsma 1974, Eller 1975/.

MATERIAL AND METHODS

Carp population of more fish farms have been regularly investigated during the past few years. In the last two years /1979-1980/ data on carps of all the three age groups of 4 fish farms were collected for comparison.

After post mortem examination on the spot, organs were fixed in 10 % formaldehyde. Samples were processed partly for freezing, partly for embedding in paraffin. For staining P. Mayer's hemalaun-eosin, Van Gieson, Giemsa, PAS methods were used. Sections were made from all gill samples both in longitudinal and transversal level. At the same time parasitological, bacteriological and virological examinations were carried out.

RESULTS

Gill changes found can be divided into 4 groups:

Stage 1 /early damages/

The gross pathology is not characteristic. In the conjuctive tissue of gill arches PAS positive acidophilic granulocytes can be seen. Epithelial cells covering foliae and lamellae do not show any alteration by light microscopy. This stage should be differentiated from damage of Myxobolus at the basis of foliae and in the interhemibranchial conjuctival tissue. These cysts sometimes cannot be discovered by native examination /Fig. 1/.

Stage 2 /acute inflammation/

Gill is swollen, many granulocytes can be found by native examination. These cells are characteristic under the light microscopy as well. Eosinophylic granulocytes can be seen in great numbers in the epithel of arches and foliae. At the base of foliae, proliferated epithel covers the filaments that degenerate later. They will be replaced by granulating tissue which adheres lamellae nearby. Hyperplasia of filaments and occasionally degeneration of the respiratory epithel is also noticeable.

Figure 1 Normal gill lamellae of a control carp. HE x 40

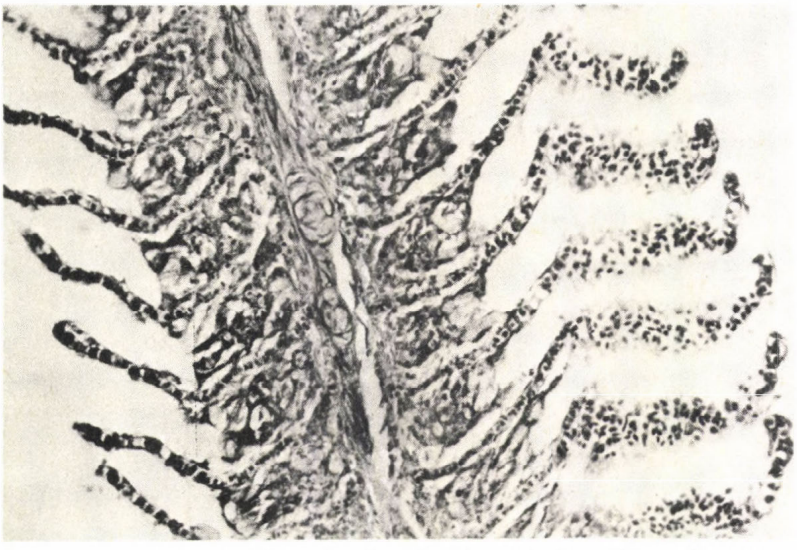

Figure 2 Normal gill lamellae to the left and lamellar hypertrophy in the right-side zone of picture. HE x250

This stage can be differentiated from the similar histopathology of anoxia, pesticide toxicity, effect of heavy metals on the basis of anamnesis and water quality control, as well as taking the results of examination of other organs into consideration /Figs. 2,3/.

Stage 3 /chronic inflammation/

Gills are greyish in colour and fewer granulocytes are present in the native smear. Tissues around the arches of the gill are covered with mucus. Sometimes Ciliata can be found in great number on the surface of the gill. Such changes occur generally at the end of winter /Fig. 4/.

Hyperplasia of gill epithelium advances toward the apical end of filaments. The epithel cells remain generally intact, but in some instances degeneration, lysis occurs accompanied by degeneration of mucous cells, eosinophilic granulocytes and haemorrhages appear.Different parasites and in one case Sanquinicola miracidia were revealed with similar changes. In case of bacterial infection, besides acidophilic granulocytes, cells of a blue shade, containing heterophyl granules also appear around the tissues of gill arches. These latter cells are PAS negative. Hyperplasia of gill epithelium is seen furthermore in the length of foliae. In case of Dactylogyrus invasion, hyperplasia starts at the proximal segments of filaments /Fig. 5/.

Mucous cells also proliferate at first, but degenerate later, causing adhesion of neighbouring lamellae. Flexibacteria and other bacteria can often be seen on the spot of alterations /Fig. 6/.

Stage 4 /necrosis/

This stage of necrosis is regarded as the most serious one. Even with the naked eye, gills are pale and have a marmour-like pattern. Because of breaking down of lamellae, the gill seems to be cogged. According to our experience all the earlier stages may turn into necrosis. Focal necrosis occurs in the 3rd stage frequently. The histological changes as hyperplasia of epithelium and necrosis starting from the base of foliae cause significant alterations in the gill structure. Diffusing capacity of the gill gradually decreases.

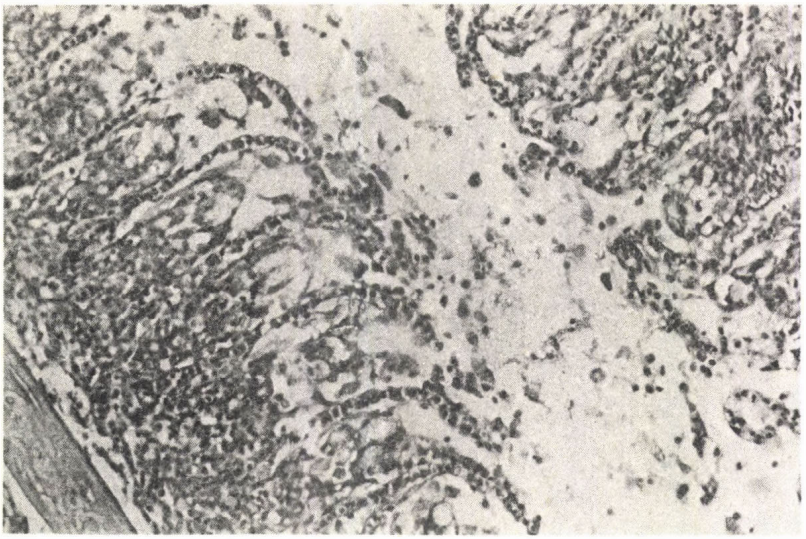

Figure 3 Hyperplasia of filaments and degeneration of respiratory epithel of gill. HE x250

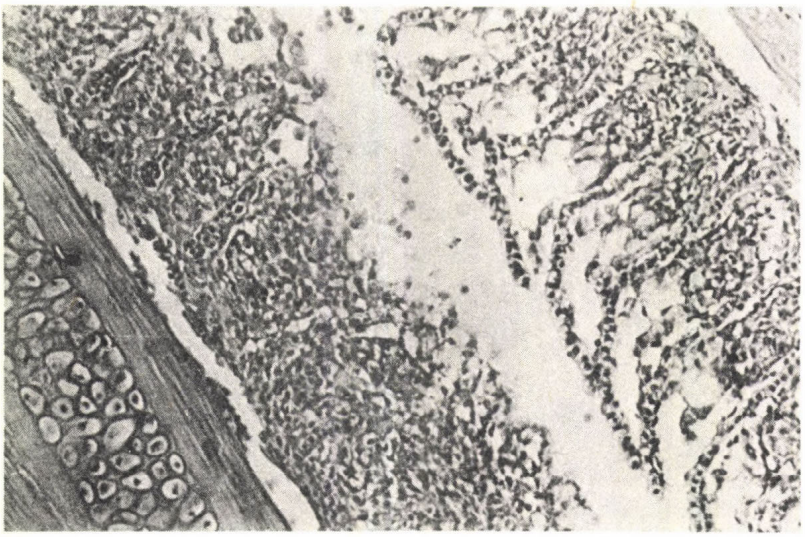

Figure 4 Focal necrosis and adhesion of neighbouring lamellae. HE x250

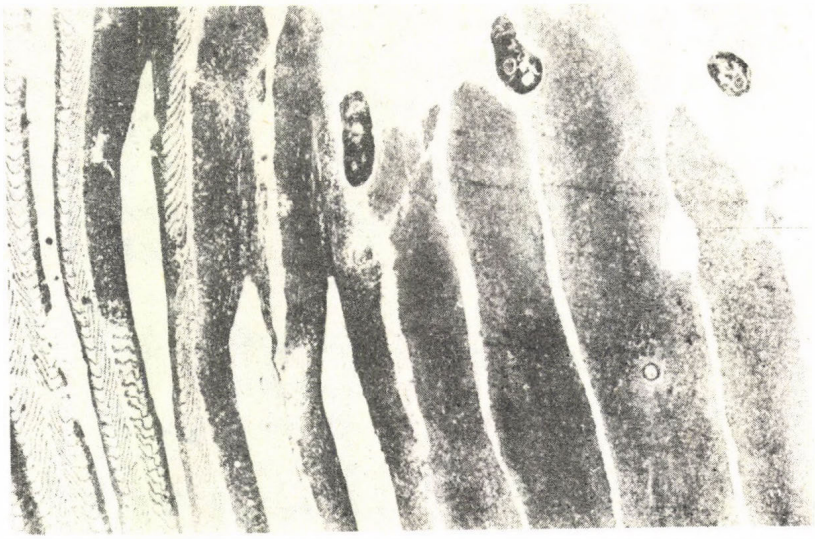

Figure 5 Necrosis of filaments with some trematodes. Giemsa x40

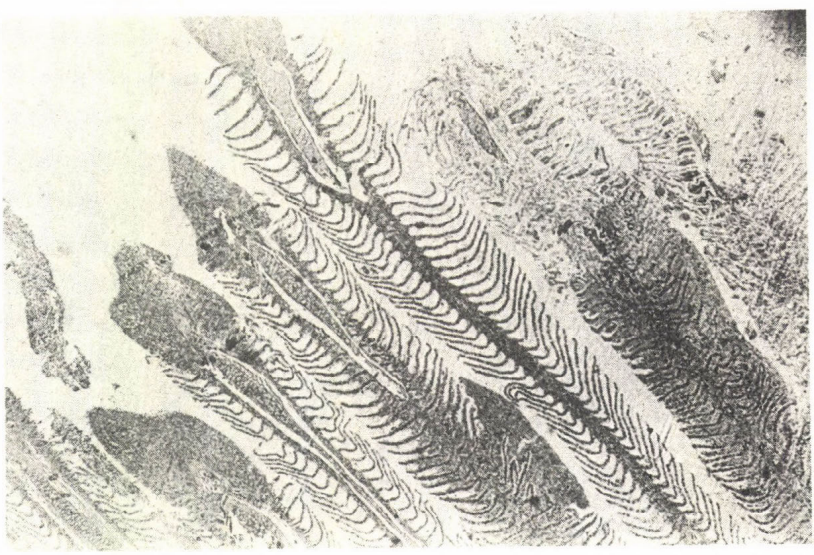

Figure 6 Focal necrosis in the apical region of gill filaments. Giemsa x40

Figure 7 Focal necrosis in the apical region of gill filaments Giemsa x240

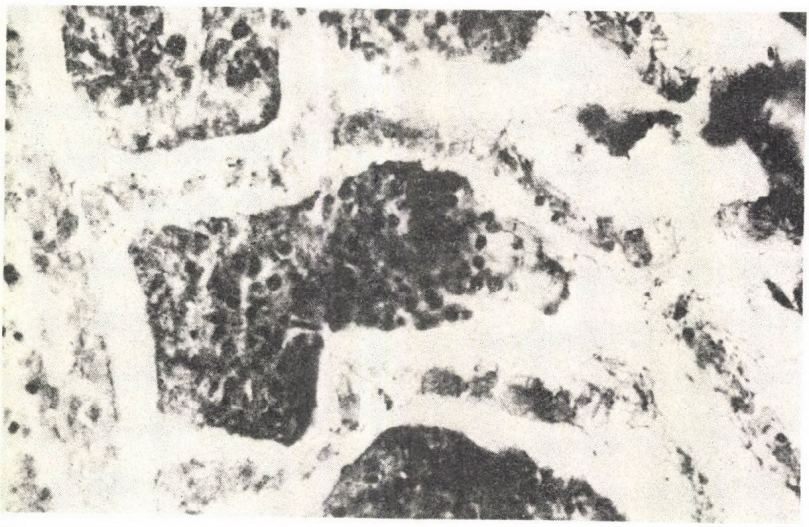

Figure 8 Flexibacteria can often be seen on the spot of pathological lesions. Giemsa x1,000

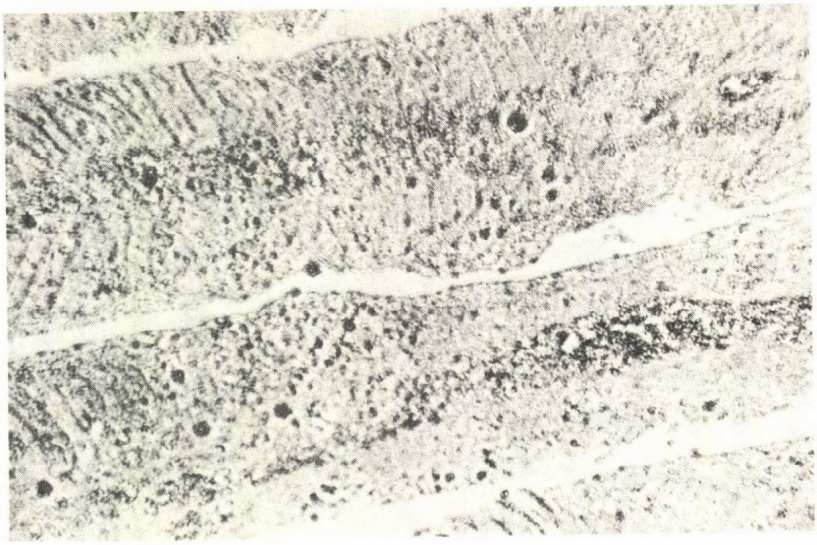

Figure 9 Special form of gill necrosis. HE x100

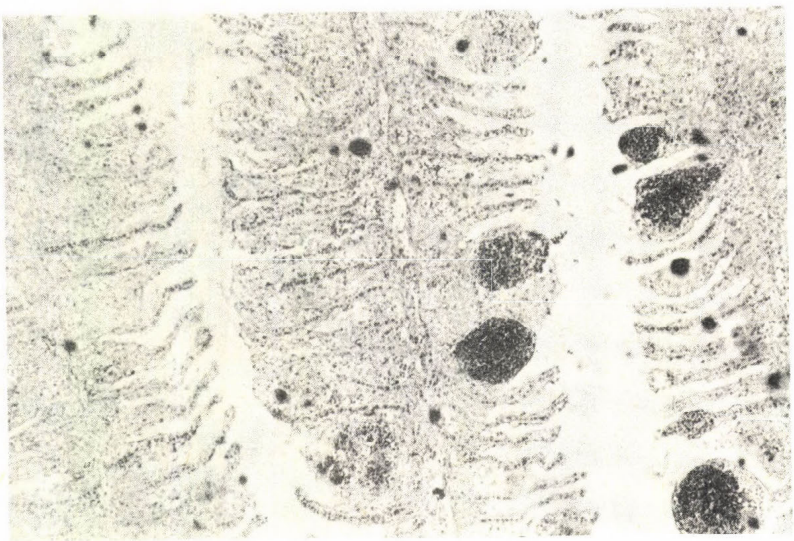

Figure 10 Hyperplasia of filaments and lamellae aneurisms of gill. HE x100

Necrosis can be aggravated by the effect of parasites and bacteria as well /Figs. 7,8/.

In 1980 some special forms of necrosis occurred where the pillar cell system seemed to be broken down, so the epithelium enclosed a disorganized mass of erythrocytes. Such changes have been described due to Mucophylus and experimental NH_3 toxicosis /Figs. 9, 10/.

DISCUSSION

Pathogens causing acute gill changes have been examined by many authors. The effect of ammonia exposure was described by Smith and Piper /1975/ and by Smart /1976/ in trout, by Flis /1968/ in carp. Chronic gill damage was seen in cases of sublethal ammonia and pesticide toxicosis published by Walsh and Ribelin /1975/ and due to the effect of Zn-ions /Skidmore 1970/. Similar lesions were found by Scott and Rogers /1980/ in hypoxia, and by Kühn and Koecke /1956/ in fish kept under conditions of high pH.

Our findings partly differ from these histological descriptions /Kovács-Gayer 1977/. Namely, the hyperplasia of respiratory epithelium due to acute ammonia toxicosis resembles the 1st and 2nd stages found by us, but the infiltration of eosinophilic granulocytes has never been mentioned by the authors. The same is true for the similarity of chronic inflammation found in gill necrosis, that is characteristic for the sublethal effect of ammonia, but such factors as the further presence of eosinophilic cells and the possibility of secondary pathogens as bacteria have not been discussed. Gill necrosis caused by Sanguinicola miracidia was described by Schlotfeldt /1980/, but the invasion has been found very rarely in our country. As far as other parasites are concerned, their effects are discussed in the work of Molnár and Szakolczai /1980/. Branchyomyces infection first described by Miaczynski /1965/ has not been confirmed during the past years.

We must stress the importance of different bacteria that can aggravate lesions of respiratory surface. This experience is in accordance with Spangenberg's statement /1975/. Histopathology is important in the diagnosis of early stages of

gill necrosis but other laboratory analyses are also needed to establish the disease. Parasites and especially bacteria can be revealed only in chronic cases in greater number. Gill damage due to Myxosporidia in the interlaminar epithelium is a new finding.

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IMMUNOLOGY

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VACCINATION OF FISH IN EUROPEAN POND CULTURE: PROSPECTS AND CONSTRAINTS

N. FIJAN

Department for Biology and Pathology of Fish and Bees,
Veterinary Faculty, University of Zagreb, Zagreb, Yugoslavia

ABSTRACT

This paper reviews briefly the data on the immunization of carp for the prevention of infectious dropsy and spring viremia. It discusses the advantages and disadvantages of the five known antigen delivery methods to fish. It also indicates gaps in the knowledge on these methods and points out some aspects of future research on immunoprophylaxis of spring viremia of carp and carp erythrodermatitis and finally assesses shortly the significance of the epizootiological situation in the pond culture for the possible benefits by immunization. In addition to the still inadequate information on etiology of several pondfish diseases and insufficient research data on immunization, one of the main constraints to a faster introduction of immunoprophylaxis into practice is the present impossibility to incorporate most of the known vaccine administration methods into the production technology at a time when the water temperatures favour a good immune response of fish.

INTRODUCTION

The main part of aquacultural fish production in the world as well as in the central, Southeastern and Eastern regions of Europe originates from the warmwater pond fish culture. In the future, the importance of pond farming will remain or even become more significant. The attempts to increase the existing fish production by various intensification approaches are under way in practically all countries. One of the obstacles to reaching high-

er yields per unit of carp pond area in Europe are the relatively high mortality rates of fish and other damages caused by diseases. The better health protection of fish in ponds is, therefore, one of the decisive factors for productional and economic progress of aquaculture.

In some forms on fish culture vaccination is apparently becoming a practically applicable prophylactic measure. Namely, the immunoprophylaxis of vibriosis in salmonids is possible and applied in several countries. The vaccination for redmouth disease of trout /yersiniosis/ is equally successful. Although the use of these vaccines did not always result in a satisfactory high degree of specific protective resistance in fish under practical conditions /Busch 1978/, it seems that rapid progress in this field can be expected in the near future. The aim of this paper is to discuss the potential significance of immunoprophylaxis for prevention of infectious diseases in the pondfish culture and to outline the main constraints to its applicability.

HISTORY

The first experiments on the immunoprophylaxis in fish culture were carried out by Schäperclaus in 1936 and during several consecutive years /Schäperclaus 1955/. In his numerous laboratory and field tests for development of vaccination against infectious dropsy of carp /IDC/, Schäperclaus applied several types of Aeromonas punctata vaccines to carp by injections or orally. The intraperitoneal immunization resulted in decreased mortality rates of carp kept in ponds /Schäperclaus 1955/ as well as in considerably higher levels of agglutinating and precipitating antibodies than in the control carp /Schäperclaus 1972/.

The vaccines prepared from Aeromonas punctata and Pseudomonas fluorescens were tested by many other authors. For instance, Lukjanenko and Sukacheva /1972/ studied the effect of single and multiple antigen injections in various doses and later /1975/ compared the immune response of four carp genotypes to such antigens. Osadchaya et al. /1972/ immunized carp with a "polyvalent" vaccine consisting of several aeromonad and pseudomonad isolates from IDC cases and challenged them by inoculation of scarified skin with pathological material from diseased fish.

Both the immunized and the unimmunized control carp developed a typical disease after the challenge in spite of the fact that the immunized fish had high titres of agglutinating antibodies against Aeromonas punctata and fluorescens bacteria.

Two works explored another approach to immunoprophylaxis of IDC. Goncharov /1951/ recommended the preparation of a tissue vaccine from the hemorrhagically inflamed skin of ill carp. In a laboratory experiment, Fijan and Cvetnich /1969/ applied this immunization procedure and found a slight protective effect when carp were challenged by application of infectious skin tissue onto scarified skin.

During the past ten years we have carried out a number of experiments in laboratory and in ponds on the possibility of preventing outbreaks of spring viremia of carp /SVC/ by vaccination /Fijan et al. 1972, 1973, 1977a, 1980/. The results can be summarized as follows:

a/ Intraperitoneal inoculation of carp with live unattenuated or partially attenuated SVC virus /Rhabdovirus carpio/ at temperatures above 19-20°C resulted in a solid and long lasting protective immunity. Fish vaccinated at the end of the summer or at the beginning of autumn and kept in ponds were resistant to challenge with the virus next spring. In one long lasting experiment the resistance was constant up to 11 months after inoculation. The neutralizing antibodies were present only in a part of these injected fish at 30 and 60 days, but not at 90 days after the vaccination.

b/ Oral application of live virus vaccine in the laboratory induced the protective immunity in carp. In one out of two small scale pond experiments the vaccinated fish were more resistant than the controls up to nine months. Less than 10 per cent of the resistant fish had neutralizing antibodies. In two of the three large-scale pond experiments, where over 0.5 million of fingerlings were vaccinated orally at the end of the summer, no mortality or symptoms of SVC occurred. In a pond, where vaccinated fish were stocked in spring together with the unvaccinated ones, SVC developed and caused slight losses.

c/ The immersion /or bath/ of fish in live virus vaccine was carried out in two laboratory and in one field experiments, respectively. The solid protective immunity was obtained in one of the laboratory experiments /Fijan and Matashin 1980/. The intraperitoneal vaccination of carp against SVC with inactivated R. carpio carried out by Tesarcik et al. /1978/ resulted in good protective immunity.

PROSPECTS AND CONSTRAINTS

The impressive contribution of vaccination in the control of infectious diseases in man has been crowned recently by the World Health Organization's announcement about the complete worldwide eradication of smallpox. In domestic animal husbandry, the immunization is an unavoidable prerequisite for the modern intensive biotechnologies and for the suppression of many economically devastating infectious diseases. Theoretically, the effective fish vaccination has the same potential.

Up to now, the immunization has not become practically applicable in carp pond culture, but there is an ever growing need for the utilization of this preventive measure. The vaccination seems to be the only practical solution for the control of viral diseases on large farms. The bacterial vaccines /bacterins/ could reduce the risks of possible problems with resistance development in fish pathogens to antibiotics as well as to secure a more economical and longer lasting control method than the use of chemotherapeutics. If the legislation on the treatment of fish with drugs becomes more restrictive /as it is in some countries/, the development of immunization will become an imperative. An additional argument for the use of the bacterins is the recent finding about the suppressive effects of oxytetracycline on the immune system of carp /van Muiswinkel 1981/. Such effects are probably exerted by some other chemotherapeutics, as well.

The impressive progress in understanding specific and non-specific defence systems in fish recently is rapidly diminishing the lag in the knowledge of the corresponding fields in mammals. Although incomplete information on fish immunology does not seem to be a major obstacle for introducing vaccination into the pond culture praxis, further information on related problems would certainly speed up the process.

1. Vaccination systems

The number of methods suggested and tested for the antigen delivery to fish has been increased to five during the past five years. In addition to the application by injection and oral administration, the hyperosmotic infiltration, direct addition to water /bath, immersion or flush treatment/ and the spray method were investigated.

Of all the immunization methods, the application of antigen by intraperitoneal injection of fish secures the strongest immune response and the longest resistance. The polyvalent vaccines could be most efficiently administered by this route. The extensive use of manpower is one of the two main obstacles to its wide use in praxis. The second and more serious problem is the impracticality of carrying out the fishing of large ponds, the safe handling of large fish numbers for injecting and their stocking at water temperatures which would enable permanent immunity to be implemented.

Oral immunization has a very important advantage: it can be carried out at high pond water temperatures which secure a good immune response. Before its dependable applicability, research will have to solve a number of questions, such as a/ the stability and effectiveness of individual vaccines and bacterins in mixtures with various feeds, b/ the mechanisms and location of antigen contact with the immunological system, c/ the effectiveness and duration of acquired resistance to bioagressors, d/ the influence of the status of organism and of epithelial contact surfaces on effectivenes and e/ the suitability of various vaccine and bacterin preparations from the same pathogen for this immunization method.

The vaccine delivery by water route has not been tested sufficiently in species reared in ponds. The obstacle to the immediate applicability of the otherwise very attractive immersion, dip or bath vaccination is the impossibility to incorporate such an operation into the present pondfish culture management practice: fish are normally not taken out from the ponds at water temperatures suitable for immunization. One exception is the fingerling rearing technology where the one-month-old fish are removed from nursery ponds. Unfortunately, at this age the carp

probably does not reach full immunological competence yet: van Loom et al. /1981/ could not find any demonstrable antibody producing cells in carp stimulated immunologically at one month of age.

The hyperosmotic immunization technique may not have any advantage over dip, bath or flush treatment. Anderson et al. /1980/ found, for instance, the hyperosmotic predips to be unnecessary for administration of Yersinia ruckeri and Aeromonas salmonicida bacterins to rainbow trout. More work is needed on this method in warmwater fish.

When describing the spray vaccination method, Gould et al. /1978/ did not find any differences in immune response of salmonids to bacterins administered by spraying with and without pressure and by immersion. According to the authors, the spray vaccination may have some advantages over immersion methods. In pond-fish culture it may reduce the amount of vaccine needed for large fish, when compared to immersion. There are no reports yet on the experiments with this method of immunization in warmwater fishes.

2. Vaccines and bacterins

As in all other animal husbandries, the warmwater fish culture will benefit first from the immunization for diseases caused by bacteria and viruses.

The research efforts on immunoprophylaxis for SVC will probably be increased in the future. Recent data from laboratory experiments of Baudouy et al. /1980/ on the high losses caused by SVC at low temperatures indicate the possible role of this disease in some of the unexplained high mortality rates among fingerlings during the winter. The confirmation of this suspicion would further increase the importance of SVC vaccination. Two serotypes of R. carpio are known now /W. Ahne personal communication/ and a bivalent vaccine may be needed for implementation of immunization in praxis.

The development of modified live vaccine for SVC seems to have the following advantages over an inactivated preparation:
a/ live modified viral vaccines used in human and veterinary medicine produce a longer lasting and more effective response;
b/ the live preparation is more suitable for administration to

fish by all vaccination methods except possibly by the injection and c/ vaccination with live virus requires a smaller dose of viral particles, thus being more economical. The widespread occurrence of SVC indicates a low epizootiological hazard by the use of live vaccine.

There are no reports yet on the attempts of the immunoprophylaxis for carp erythrodermatitis. The frequent and widespread occurrence of this disease as well as the high cost of prevention and treatments by chemotherapeutics would certainly justify the introduction of immunization. The initial localization of the infection on the skin may cause the evoking of a solid protective immunity to be a difficult task.

To our possibly biassed opinion, the immunization against Aeromonas punctata and related bacteria is not of great practical significance. More urgent problems are the diseases of unexplained or disputable etiology, such as swim bladder inflammation, gill necrosis and the infectious diseases of other cyprinids reared in carp ponds.

3. The epizootiological situation on pondfish farms and the immunoprophylaxis

Fish mortalities in European carp ponds are caused by several diseases which sometimes occur simultaneously in the same population or even in the same fish. In such epizootiological situation no profound improvement of the health status can be expected from the introduction of the immunization for one single disease. Even the best polyvalent vaccines will not replace the need for better use of fish health protection measures, for their incorporation into all technological operations, for correcting the adverse environmental conditions and for the avoidance of other stress factors. However, in a well managed farm, a good immunoprophylaxis could certainly reduce the risks and losses as well as increase the production.

CONCLUSION

The immunoprophylaxis for the diseases of fishes reared in European ponds has so far received relatively little attention. The new vaccine delivery methods developed for salmonids and

the progress in knowledge on the etiology of some diseases have opened new research possibilities on immunization for two carp diseases of major importance.

The largest constraint to applicability of immunization is the present impossibility to use in praxis, four of the presently known five vaccine administration techniques to immunologically competent fish at water temperatures that favour a good immune response. If the oral immunization does not prove to be effective, the present pond farming technology may have to be modified so as to be able to apply other vaccination methods at temperatures above 15°C.

The epizootiological situation in large ponds favours the development of polyvalent immunizing preparations. It also indicates that immunoprophylaxis may significantly reduce the losses, but it cannot replace other disease prevention measures.

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QUANTITATIVE INVESTIGATION OF NATURAL HEMAGGLUTININS TO HUMAN ABO BLOOD GROUPS IN THE SERA OF THREE FRESHWATER FISHES

J. SÖVÉNYI, I. PETRI*

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest
*Blood Transfusion Centre, Szeged, Hungary

ABSTRACT

Sera of hatchery-reared carp, common bream, pike-perch from Lake Balaton were examined for the presence and activity of natural hemagglutinins to human ABO blood groups.

54.5 % of the serum samples of carp, 38.7 % from common bream sera and 95.6 % of pike perch's sera agglutinated at least one red blood cell suspension of the four blood groups.

Average titre values obtained in the three species were low /carp: 30.31, bream 9.20, pike perch 20.31/.

Average titres of the sera decreased in the order of O, A, B, AB blood groups in case of carp, - O, B, A, AB blood groups in bream and this order was B, AB, O, A in pike perch.

Positive correlation was found between the average titre values obtained with different blood groups in carp.

The authors presume that carps and white breams examined may have been in close connection with some bacteria bearing an H-antigen-like molecule.

INTRODUCTION

Agglutinating activity of the calf serum against human erythrocytes has been known since 1927; and the same effect of the eel serum has been discovered decades ago. Since then, a large number of papers have reported cross-reactions between human erythrocytes and sera of different fishes /Baldo 1972 a, b, 1973, Legler et al. 1971, Holt and Anstee 1975/.

We do not know yet what the origin of these cross-reactions is exactly. What we know is, that the surface antigens on the human erythrocytes contain some defined heterosaccharides. The natural hemagglutinins react with these determinants and the reaction can be inhibited by absorption with the suitable sugar residues, except for brown trout /Baldo 1972a, 1972c, Holt and Anstee 1975/.

The natural hemagglutinins in the fish sera are usually proteins with high molecular weight. Their antibody character has not yet been proved satisfactorily /Baldo 1973, Legler et al. 1971, Salák et al. 1975/. They may be present in the thymus and the spleen as well /Vikhman 1979/. Most of them are heat stable and preserve their activity after incubation at 56°C for 30 min.

The appearance of natural hemagglutinins in the organism depends on the age and environmental effects among which the habitat and some diseases causing strong immune response have role /Vikhman et al. 1975/. Heterosaccharides of blood-group antigens are common in antigens of bacterium strains widespread all over the world. Immunizing with certain bacterial antigens, we can also enhance the natural hemagglutinins /Legler et al. 1971/.

Grubb /1949/ having immunized eels with H-antigen characteristic for the human O blood group, got a serum active against blood group O, A_2, B, A_1, A_1B. The titre values decreased in the same sequence.

Our aim was to establish the presence and frequency of natural hemagglutinins in the blood of white bream and pike perch. Blood sera of these fishes have not been investigated yet in respect of natural hemagglutinins. On the other hand, we tried to find a correlation between the titre values obtained with different blood-group antigens. We presumed, that we might learn more about the antigen/s/ stimulating the fish to produce natural hemagglutinins.

MATERIAL AND METHODS

Mirror carp /Cyprinus carpio L./ with a body weight of 0.6-1.1 kg were fished from the ponds of Fish Farm of Fehértó /Szeged/. Blood was taken 15 min /at most 25 min/ after

fishing. Blood samples of group carp I /42 specimens/ were collected in April, and those of group carp II /88 specimens/ in July.

White bream /Abramis brama/and pike perch /Stizostedion lucioperca/ were caught in May in Lake Balaton. Their weights were 0.4-0.7 and 0.8-3.2 kg, respectively. Blood samples were taken after harvesting at once.

The coccigeal arteria was punctured. Blood samples were allowed to clot for an hour at room temperature and stored at 40°C, for 24 hrs. Sera were collected; inactivation was made by incubation at 56°C for 30 mins.

Sera of the test group carp I were checked by slide-agglutination. Ten red-blood cell suspensions from different donors were added to every serum sample in the four blood-groups each, respectively. Human red-blood cells were washed three times, and suspended in 0.9 % NaCl solution to make a 1.5 % suspension. Agglutination was judged after 3-5 min incubation at room temperature. A certain serum was considered to be positive in a respect if it agglutinated all the red blood cell suspensions /RBCS/ of a certain blood group. Titre values have not been measured.

Slide agglutinations of the test groups carp II, white bream and pike perch were carried out using a mixture of aliquots of red blood cells originating from ten persons in each blood group, respectively. Density of the RBCS was adjusted to 0.5 % with hematocrit measure.RBCS and serum dilutions for titration were made with Dulbecco's PBS /+0.02 NaN_3/. Positivity was titrated on Takátsy's microtitre plate. Sera were diluted in two serials. One was a two-fold serial, the other started from a three-fold dilution followed by the usual two-fold steps. Equal amounts of RBCS were added to the dilutions. Titre values were read after an hour incubation at room temperature. Reciprocals of the last agglutinating dilutions were taken as titre values.

Regression of the titre value-pairs were fit to nine linear and non-linear equations.

RESULTS

Hemagglutinins of different specificity occur mostly together in a certain specimen if they appear at all /Table 1/.

There is no characteristic difference between the frequency of positive reactions with different blood-group antigens concerning the sera of carp and white bream. Among the pike perch sera there were a relatively few agglutinating A type RBCS /Table 2/.

Table 1 Distribution of fish according to the number of positive reactions

Species	No agglutination n	%	Agglutination with 1 type of RBCS	2	3	4	Number of positive sera n	%
Carp I-II /n=130/	56	43.1	5	9	20	40	74	56.9
White bream /n=31/	18	58.1	1	1	4	7	13	41.9
Pike perch /n=23/	1	4.3	0	0	6	16	22	95.7

Table 2 Number of positive reactions with different type RBCS

Species	O	A	B	AB
Carp I-II	58	66	57	61
White bream	11	11	11	10
Pike perch	22	16	22	22

Hemagglutinins rarely occur alone in a given specimen. Among the carp sera reacting with three kinds of RBCS, the common occurrence of anti-O,-B,-AB heterohemagglutinins was the most frequent /Table 3/.

Heterohemagglutinin titre values were low in all the three species. Mean titre values diverged from each other to the least extent in the white bream, while there was a three-fold difference between mean titre values of carp taking blood groups A and AB. Deviation of the means was relatively high. There was a discrepancy between the species in respect of the order of magnitude of mean titre values /Table 4/.

Table 3 Distribution of fish specimens according to the occurrence of different types of heterohemagglutinins

Specificity of hemagglutinins found	Carp I-II	White bream	Pike perch
only O	-	-	-
only A	3	-	-
only B	1	1	-
only AB	1	-	-
O-A	2	1	-
O-B	2	-	-
O-AB	-	-	-
A-B	2	-	-
A-AB	1	-	-
B-AB	2	-	-
O-A-B	3	1	-
O-B-AB	2	2	6
A-B-AB	6	1	-
O-A-AB	9	-	-
O-A-B-AB	40	7	21

Table 4 Mean titre values /$\bar{x}$/ of heterohemagglutinins, and deviation of the means /$S\bar{x}$/

Species	O	A	B	AB
Carp I-II				
n	41	44	42	47
$\bar{x}$	30.31	21.18	14.88	10.14
$S\bar{x}$	±8.43	±6.71	±5.33	±2.77
White bream				
n	11	11	11	10
$\bar{x}$	9.20	5.72	6.18	5.70
$S\bar{x}$	±2.55	±0.85	±0.90	±0.92
Pike perch				
n	22	16	22	22
$\bar{x}$	6.22	5.93	20.31	20.00
$S\bar{x}$	±0.59	±1.33	±3.33	±3.03

Regression of titre value-pairs of different specificity in carp could be expressed with the equation: $Y' = a + bX$. Correlation between titre values of any possible relation was strong, and between the titre values obtained with blood groups B, and AB was very strong. Distribution of titre values of the other two species could not be defined satisfactorily, because of the relatively low number of specimens.

The difference between the mean titre values is significant on a $p=0.05$ level except for the relations of means of A-O, and A-B specificity /Table 5/.

Table 5 Correlation /r/ between titre values obtained with different blood groups and significance /s/ of the difference between the mean titres in test group carp II

Carp II	O-A	O-B	O-AB	A-B	A-AB	B-AB
n	48	48	47	47	47	48
r	0.776	0.870	0.814	0.814	0.834	0.951
s	$0.09 \geq p \geq 0.08$	$p < 0.05$	$p < 0.05$	$p \geq 0.1$	$p < 0.05$	$p < 0.05$

DISCUSSION

Frequency of heterohemaglutinins in the three species does not show extreme values compared to the data published by others /Baldo 1972 b, Holt and Anstee 1975, Legler et al. 1970, Legler et al. 1971/. Almost every pike perch serum agglutinated some of the blood group substances in this way representing a relatively rare case.

Mean titres of the carp divert only slightly from those described by Baldo /1972 b/ for carps in Australia. There are no data on heterohemaglutinins of white bream and pike perch either.

Heterohemagglutinins of different specificity /O,A,B,AB/ appear mostly together in the specimens that bear them at all. This does not allow us to suppose that they should originate from contact between the organism and several antigens of various characteristics. In this case we would find mostly fish with heterohemagglutinins of one specificity while scarcely those possessing all kinds. Strong correlation between the titre values

of diverse heterohemagglutinins in carp serum samples also contradict the idea of independent production of heterohemagglutinins, i.e. the idea that they originate generally from separate antigens.

Grubb /1949/ has proved with his hyperimmunization experiments that the eel serum produced against a single /H/ blood-group antigen agglutinated red blood cells of other specificities too. Titre values can be arranged in an other order of O, O, A_2, B, A_1, A_1B according to their decrease.

In the present paper we did not differentiate between the blood groups A_1 and A_2. But the decreasing order of mean titre values of carp and white bream is very similar to that described above: carp - O, A, B, AB; white bream - O, B, A, AB.

On the basis of this similarity, we think, that these two species may be in a close and steady contact with bacteria bearing an H-antigen-like substance. This assumption, however, needs further data to be proved.

Results with pike perch sera cannot be regarded in the same way, as the decreasing order of the mean titre values is quite different from that of the other two. Several factors could be considered as a cause. The percid pike perch is a predator while the two cyprinids find their nutrients mainly in the sediment. It is also natural, that the characteristics depending on species have also an influence on the interaction betwen the organism and the antigens. Many missing data inhibit proper explanation of the sophisticated relation which - during this interaction - reflects, at the same time, the differences of both the fish species and the antigens.

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FISH BIOLOGICS: ANTISERA FOR FISH DISEASE DIAGNOSIS

D.P. ANDERSON, O.W. DIXON

U.S. Fish and Wildlife Service, National Fish Health Research Laboratory, Kearneysville, West Virginia, 25430 USA

ABSTRACT

The production and distribution of biologics used for detection, identification, and prevention of fish disease agents has enabled serodiagnostic methods and immunization to become valuable tools for fish pathologists. The immunization regimens, including schedules of antigen exposure, doses, and choice of adjuvant used for rabbits or other animals, may differ for the particular disease agent. The quality of the final antisera may be dependent not only upon titer, but on the presence of cross-reactive antibody. This paper outlines some of the procedures used for antisera production at the National Fish Health Research Laboratory.

INTRODUCTION

Serodiagnostic methods for fish disease agent detection and identification have become important tools for fish pathologists as antisera, antigens, and other reagents are made available /Dixon 1978; Anderson et al. 1980/. The production and maintenance of standardized stock of biologics present special problems because of the undefined nature. Antisera are especially troublesome because of individual variation among individual animals used in production.

There has long been recognized a need for high-quality antisera for fish biologics that will have high titers and demonstrate little or no cross-reactivity with unrelated pathogens.

When antisera is produced, certain compromises must be made when selecting an immunization schedule. Capability and demands of staff expertise, avoidance of discomfort to the animal, expense involved in the total schedule of antigen preparation, time of administering immunogens, and limitations of animal facilities are some of the factors which might affect the immunization schedule selected. In addition, each immunogen has intrinsic characteristics that will determine factors of the schedule.

In this paper we describe some of the materials, methods and results of the immunization regimens used for the production of antisera against fish disease agents in the Biologics Section of the National Fish Health Research Laboratory during the last 7 years. Many facets of these regimens are subjective /i.e. doses, use of adjuvants, timing of injections, etc./; therefore, we can only recommend our experience as a guide for others.

MATERIALS AND METHODS

Animals

The majority of the antisera are produced in New Zealand white rabbits 3 to 6 months old, weighing 1.6-1.8 kg. They are purchased from Rocky Hill Rabbitry, Kearneysville, West Virginia and maintained in a constant temperature, air-controlled room with an automatic water system, and fed a ration of commercial pellets /Zeigler Brothers, Inc.[1/] Gardners, Pennsylvania/. Rabbits are monitored daily to ensure proper care.

Upon receipt in the laboratory, ears of the rabbits are tattooed and India ink applied for permanent identification. This number is also used for the resultant antisera cataloging.

Control rabbit sera for the Biologics File consists of a pooled batch of lyophilized sera purchased from Pel-Freeze Biologicals, Rogers, Arkansas. The pooled normal serum has been tested and found to be free of factors that might interfere with the recommended immunological tests.

Immunization routes

Standard routes for immunization of rabbits for antisera production are intravenous /i.v./, intramuscular /i.m./, intra-

[1/] Reference to trade name does not imply Government endorsement of commercial product.

peritoneal /i.p./, intraabdominal /i.a./, intradermal /i.d./, subcutaneous /s.c./, and foot pad.

Doses

Bacterial doses given to rabbits are usually calibrated by spectrophotometer. Doses are standardized at 40 % T 5/25 μ; when heavier doses are judged necessary, the dose may be determined by centrifuged cell pack or dry weight. A bacterial counting chamber /Petroff-Hauser/ is sometimes used to enumerate individual cells in a suspension. Standardization by the McFarland scale, a less accurate method of calibrating a bacterial suspension, is done by comparing the opacity to that of standard tubes containing graded concentrations of barium sulfate /Kwapinski 1965/.

Protozoan concentrations are usually determined by direct cell count in a hemacytometer. If possible, the protein and/or lipid content is also recorded.

Doses for injection of viral agents are based on plaque forming units /PFU/ that gives an estimate of the numbers of viral particles in the suspension. Titration of virus inocula can also be determined through a tissue culture infectious dose 50 % /$TCID_{50}$/.

Soluble antigens usually require dry weight determination as is done for the bacterial O-antigens we used. Standardization is also done by spectrophotometer reading for special solutions.

Adjuvants

Complete Freund adjuvant /CFA/ and incomplete Freund adjuvant /ICFA/ are the two adjuvants most commonly used in routine immunization regimens. Other adjuvants that have been used include: alum precipitation of the antigens, the addition of mineral oil, and bentonite particle absorption of antigen.

Immunization schedules

The regimens currently used in the Biologics Section at the National Fish Health Research Laboratory are shown schematically in Figs 1 and 2.

The timing of injections in Immunization Regimen Number 1 shows that 2 days after the prebleeding sample the animal is given equal doses of antigen i.m. with adjuvant every other day for three injections. After a 12-day rest period, the booster

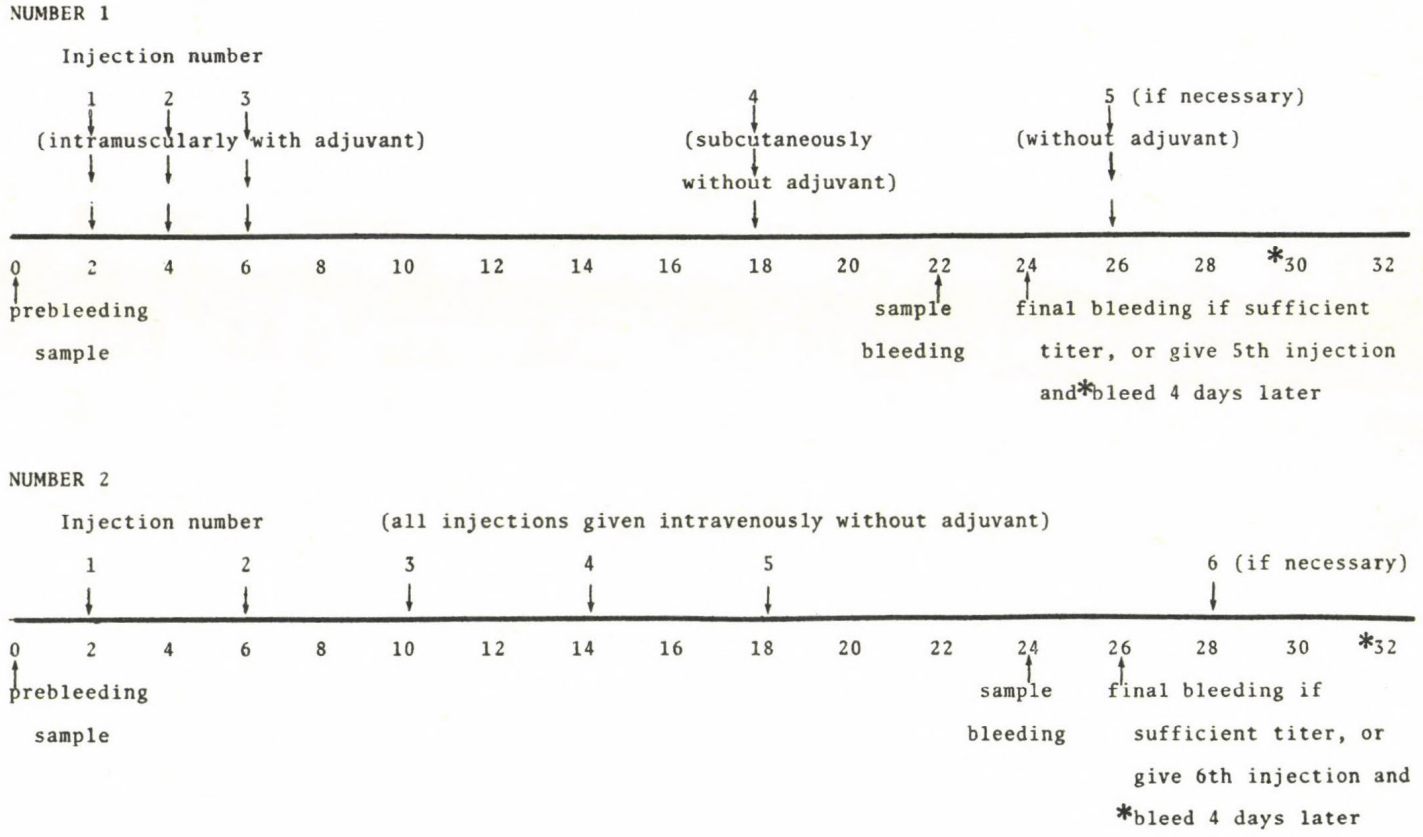
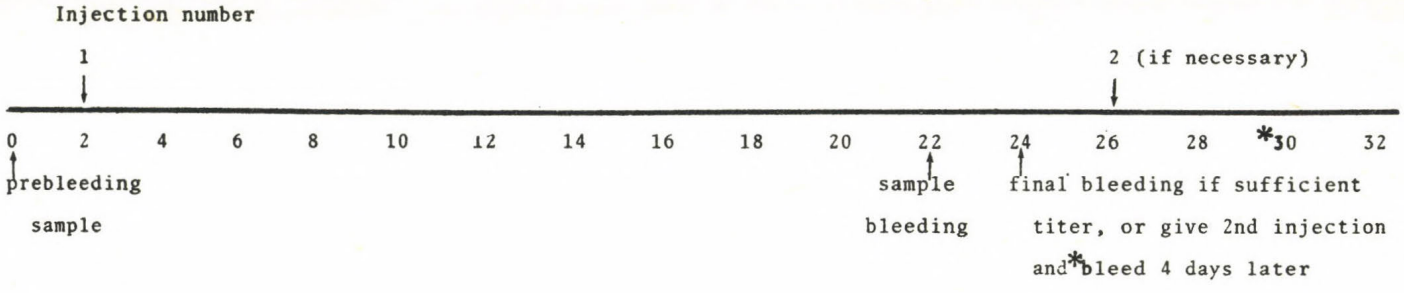


Figure 1 Immunization Schedules No. 1 and 2 used for Injecting Rabbits with Fish Disease Agents /time in days/

NUMBER 3

Multiple sites of injection given simultaneously; i.e. intramuscularly, subcutaneously, and intraperitoneally with adjuvant and intravenously without adjuvant



NUMBER 4

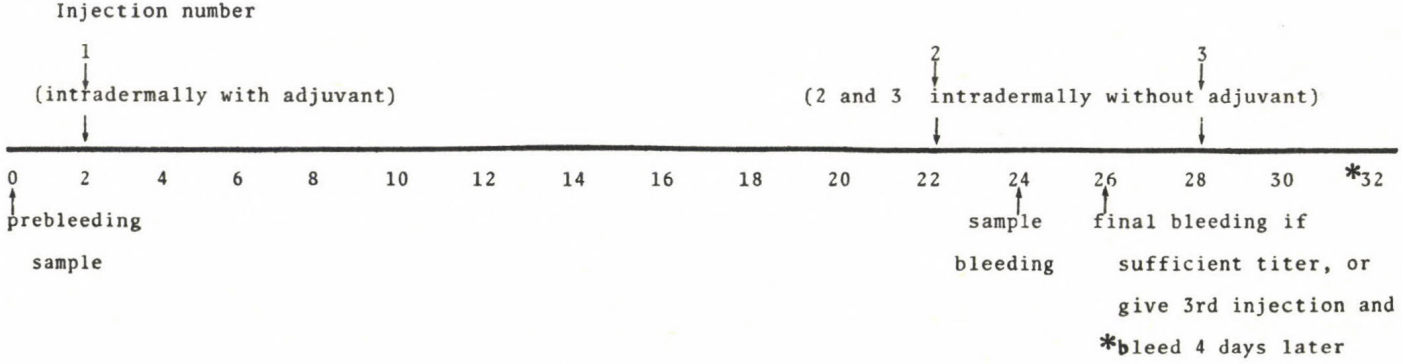


Figure 2 Immunization Schedules No. 3 and 4 used for Injecting Rabbits with Fish Disease Agents /time in days/

injection is given s.c. without adjuvant, and 4 days later, a sample bleeding is taken. If the serological test shows that the antibody titer is sufficient the animal is bled within 2 days; if not, the animal can be reinjected.

Immunization Schedule Number 2, developed by Edwards and Ewing /1972/, shows increasing doses of antigen given i.v. five times, 4 days apart. Recommended injections of 0.4, 0.8, 1.6, 3.2, and 6.4 mg are given without adjuvant. As above, a sample bleeding is taken to determine time of final bleeding.

In immunization Schedule Numbers 3 and 4, the animals are minimally handled. Number 3 shows the animal is given multiple site injections simultaneously, i.m., s.c., and i.p., with adjuvant and i.v. without adjuvant. Number 4 shows a simple single i.d. injection with adjuvant.

RESULTS

Qualified personnel may obtain the following antisera from the Biologics Section of the National Fish Health Laboratory--Leetown: /U.S. Fish and Wildlife Service Biologics are for in-house fish disease serodiagnostic tests and are not designated as standards/.

Antisera

Viral

Infectious pancreatic necrosis virus--polyvalent

Bacterial

Aeromonas salmonicida
Cytophaga psychrophila
Edwardsiella tarda
Flexibacter columnaris
Pasteurella sp.
Renibacterium salmoninarum
Vibrio anguillarum
Type I /#775/
Type II /#1669/
Yersinia ruckeri
Type I /Hagerman/
Type II /Oregon/

Reference control

Rabbit normal

Rainbow trout normal

Conjugated FITC

Aeromonas salmonicida

Edwardsiella tarda

Rabbit normal

Renibacterium salmoninarum

Vibrio anguillarum

The polyvalent antisera against infectious pancreatic necrosis virus includes antisera against five serotypes. A beginning dilution of 1/10 is recommended for virus concentrations of 10^8 PFU/ml for neutralization tests.

Agglutinating titers of antisera to the particulate formalin-killed homologous bacterial antigens range from 1/512 to 1/65,536, depending on the particular preparation in the Biologics File. Agglutinating titers are usually in the lower ranges. Information on precipitins, indirect FAT, etc., are included in the File records as various tests are done by the National Fish Health Research Laboratory personnel and others. In a few cases, there may be special problems with cross-reactions with heterologous species and strains. For instance, an Aeromonas salmonicida antisera has been shown to occasionally cross-react with A. hydrophila isolates. No cross-reactions have been reported between the two respective biotypes of Yersinia ruckeri or Vibrio species.

The reference control rabbit normal sera has no interfering cross-reactins with either viral or bacterial fish pathogens. No false positives have been observed.

The conjugated antisera may degrade with processing, lyophilization, and prolonged storage. A general working dilution is recommended on individual vials and usually ranges between 1/20 and 1/80

DISCUSSION

To provide antisera that are more specific and have greater antibody titers the present immunization regimens are selected and modified as experiments are proven. In our experience, using

limited numbers of out-bred animals, the variation among individual immune responses from the animals may vary greater than the differences among the immunization regimens; good statistical data is difficult to obtain.

At the National Fish Health Research Laboratory, New Zealand albino rabbits are the most common animal used for the production of antisera against fish disease agents. These animals are relatively easy to hold, docile, and respond well to the immunization program. Our animals are from a local rabbitry; females are preferred because they seem to give a higher antibody response. The local source is convient. In-bred strains are considered impractical at this time. Occasional disease problems such as ear mites /Psoroptes cuniculi/ and diarrhea, have occurred. The animals are treated, if necessary, and carefully monitored according to Weisbroth et al. /1974/. For large volumes of antisera, goats, sheep, or horses have been used for studies in fish disease research. When the volume of antisera is not a factor, mice,rats, or other small animals might be considered.

It has been difficult to determine which route of injection is more effective. Some investigators believe that the more irritation to the animals the better the immune response; this has not been the case in our experience. For convenience we use i.m., i.p., or s.c. routes with particulate /bacterial/ antigens, and i.v. when injecting soluble antigens.

Doses of immunogen should be carefully quantified, those that are too small may result in minimal immune response or none at all. Larger doses may result in discomfort to the animal, occasionally in the form of anaphylaxis, development of lesions, or even death.

Complete and incomplete Freund's adjuvants have a definite advantage in heightening the immune response of the rabbits to the fish disease agents; however, occasionally lesions or granulomas will occur at the injection site. In addition, while higher antibody titers may result when the animals are injected with adjuvants, the antibody spectrum is broader, thus the antisera is less specific and may demonstrate interfering cross-reactions in the serological tests.

Immunization Schedule Number 1 is most commonly used at the National Fish Health Research Laboratory with the particulate bacterial antigens such as formalin-killed Aeromonas salmonicida cells. If the antiserum is to be used for research purposes and cross-reaction may interfere with sensitive results the injections are given without adjuvant. When soluble antigens are injected, Immunization Schedule Number 2 is most often used. The increasing doses may allow for the heightening of the immune response without the development of lesions. In our laboratory, the O-antigen of the gram-negative isolates are injected using Immunization Schedule Number 2.

When minimal animal handling is required, Immunization Schedule Numbers 3 and 4 are used. Number 3 usually involves injecting massive doses with the risk of harming the animals; whereas, Number 4 involves minimal disturbance. Number 3 is more often used with large animals, such as goats.

In our experience, using out-bred animals, the variation among individual immune responses from animals may vary greater that differences among immunization regimens; therefore, recommendation of an individual regimen is tenuous.

During the last 7 years over 4,000 vials of antisera, antigens, and other reagents for serodiagnostic methods have been distributed by the Biologics Section to qualified personnel /Figure 3/. Most of the biologics are antisera against bacterial agents; with an increasing demand for conjugated antisera for use with fluorescent antibody technique. Antisera supplies against the viral agent infectious pancreatic necrosis are adequate and relatively easy to produce. Antisera against fish disease rhabdovirus agents, such as infectious hematopoietic necrosis and viral hemorrhagic septicemia, seem more difficult to stimulate; therefore, only limited quantities of the latter two are held at our Laboratory. One of the greatest problems with application of antisera to the field and research has been the definition of serotypes and/or cross-reactivity. As more specific antibody is produced, perhaps greater definition of the pathogens can be made. As our research and field practice leads to more information, revisions are made in the biologics protocols and incorporated in the Biologics Guide.

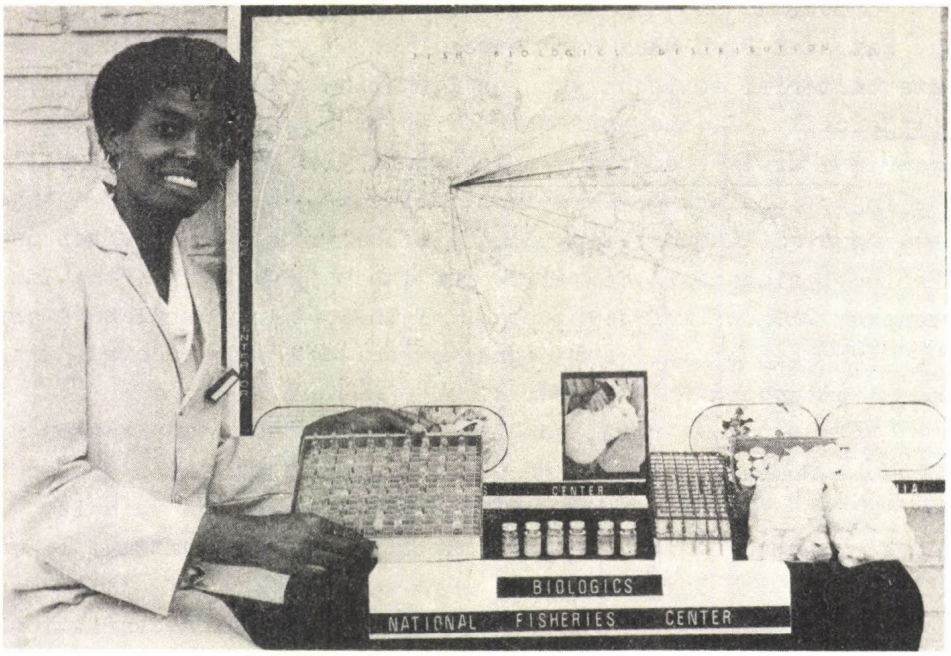


Figure 3 Samples of lyophilized antisera and antigens prepared at the National Fish Health Research Laboratory are distributed to qualified field and research personnel throughout the world for fish disease diagnosis

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SUBJECT INDEX